3HP tolerance

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(54) Title: 3HP TOLERANCE

(57) Abstract: Cells and cell cultures are provided that have improved tolerance to 3-hydroxypropionic acid (3HP). Genetic modifications to provide a mutated or overexpressed SFA1 gene or other enhancement of 3HP detoxification via a glutathione- dependent dehydrogenase reaction, including medium supplementation with glutathione, may be combined with a 3HP producing metabolic pathway.

3HP Tolerance

Field of the invention

The present invention relates to microbial cells, for instance cells of yeast strains, that show tolerance to 3-hydroxypropionic acid (3HP), including cells which produce 3HP.

Background

3HP has been produced by metabolically engineered strains of *E. coli*, see for instance WO2011/038364, EP2505656 and WO02/42418 and increased tolerance to 3HP thereby produced has been sought. Production of 3HP in yeast has also been described, see WO2012/019175.

3HP is toxic to *S. cerevisiae* and this limits the ability to produce 3HP using a metabolically engineered *S. cerevisiae*. It would be desirable to produce yeasts generally and *S. cerevisiae* strains especially that are resistant to the toxic effects of 3HP so as to provide a starting point for further genetic modification to provide an operative metabolic pathway for the production of 3HP. It would also be desirable to provide yeasts which do produce 3HP and which have enhanced resistance to its toxicity. Such 3HP tolerant yeasts can provide the basis for industrial production of 3HP by cultivation of yeasts.

The *SFA1* gene (alternatively named *ADH5*) of *Saccharomyces cerevisiae* in its wild type form has the gene sequence of SEQ ID NO:1. The gene is thought to encode an S-(hydroxymethyl)glutathione dehydrogenase.

It has been reported that Sfa1p is a member of the class III alcohol dehydrogenases (EC:1.1.1.284), which are bifunctional enzymes containing both alcohol dehydrogenase and glutathione-dependent formaldehyde dehydrogenase activities. The glutathione-dependent formaldehyde dehydrogenase activity of Sfa1p is required

for the detoxification of formaldehyde, and the alcohol dehydrogenase activity of Sfa1p can catalyze the final reactions in phenylalanine and tryptophan degradation. Sfa1p is also able to act as a hydroxymethylfurfural (HMF) reductase and catabolize HMF, a compound formed in the production of certain biofuels. Sfa1p has been localized to the cytoplasm and the mitochondria, and can act on a variety of substrates, including S-hydroxymethylglutathione, phenylacetaldehyde, indole acetaldehyde, octanol, 10-hydroxydecanoic acid, 12-hydroxydodecanoic acid, and S-nitrosoglutathione.

The five ethanol dehydrogenases (Adh1p, Adh2p, Adh3p, Adh4p, and Adh5p) as well as the bifunctional enzyme Sfa1p are also involved in the production of fusel alcohols during fermentation. Fusel alcohols are end products of amino acid catabolism (of valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, and tyrosine) via the Ehrlich pathway and contribute to the flavour and aroma of yeast-fermented foods and beverages. They may also have physiological roles. For example, exposing cells to isoamyl alcohol, derived from catabolism of leucine, stimulates filamentous growth. Similarly, other fusel alcohols also stimulate filamentous growth in *S. cerevisiae* and biofilm formation in the pathogens *Candida albicans* and *Candida dubliniensis*.

Transcription of SFA1 is controlled by Sko1p, a negative regulator of the Hog1p transcription regulation pathway. SFA1 is induced in sko1 null mutants and in cells overproducing the transcription factor Yap1p. Sfa1p expression is also induced by chemicals such as formaldehyde, ethanol and methyl methanesulfonate. sfa1 null mutants are viable and display hypersensitivity to formaldehyde, whereas overproduction of Sfa1p results in increased resistance to formaldehyde.

Sfa1p displays similarity to Adh1p, Adh2p, Adh3p and Adh5p, and to the alcohol dehydrogenases of *Escherichia coli, Schizosaccharomyces pombe, Kluyveromyces marxianus, Kluyveromyces lactis, Candida albicans, Candida maltosa*, horse, rat, and mouse, as well as human *ADH2* and *ADH3*, which are associated with the development of Parkinson disease. Sfa1p also exhibits similarity to

the glutathione-dependent formaldehyde dehydrogenase of *Arabidopsis* (FALDH), which is able to complement the formaldehyde-hypersensitivity defects of sfa1 null mutants. Sfa1p is also similar to the glutathione-dependent formaldehyde dehydrogenases of mouse and human (*ADH5*), which are involved in the catabolism of S-nitrosoglutathione, a type of S-nitrosothiol central to signal transduction and host defence.

Formaldehyde is formed by oxidative demethylation reactions in many plants and methylotrophic organisms, but *Saccharomyces cerevisiae* is a non-methylotrophic yeast and cannot metabolize methanol to formaldehyde. However, *S. cerevisiae* is exposed to exogenous formaldehyde from plant material or in polluted air and water.

Concentrations of formaldehyde of 1 mM or higher are cytostatic or cytotoxic to haploid wild-type cells. Any free formaldehyde in vivo spontaneously reacts with glutathione to form S-hydroxymethylglutathione. The level of enzymes involved in the degradation of formaldehyde, such as Sfa1p and Yjl068Cp, determine the level of formaldehyde toxicity, and cells overproducing Sfa1p are resistant to formaldehyde and null mutants in either sfa1 or yjl068c are hypersensitive to formaldehyde. Sfa1p is induced in response to chemicals such as formaldehyde (FA), ethanol and methyl methanesulphonate, and Yjl068Cp is also induced in response to chemical stresses. Molin and Blomberg, Molecular Microbiology (2006) 60(4), 925-938 reported that *SFA1* overexpression enhanced formaldehyde tolerance in *S. cerevisiae*. They reported also that supplementation of a culture medium with glutathione restored DHA sensitivity of a *gsh1Δ* strain.

Formate dehydrogenase is encoded by *FDH1/YOR388C* and *FDH2*. In some strain backgrounds of *S. cerevisiae*, *FDH2* is encoded by a continuous open reading frame comprised of YPL275W and YPL276W. However, in the systematic sequence of S288C, *FDH2* is represented by these two separate open reading frames due to an in frame stop codon.

It has been reported that the effect of certain mutations in *SFA1* or *SFA1* deletion has been to decrease resistance to formaldehyde, S-nitrosoglutathione, and peroxynitrite (Fernández,et al, 1999).

There would on this basis appear to be no known reason why certain mutations in *SFA1*, or its overexpression should be expected to improve 3HP tolerance.

We have found that a genetic modification providing overexpression of *SFA1* or providing certain mutations of *SFA1* increases the ability of yeast and other cells to grow in the presence of normally inhibitory concentrations of 3HP. Furthermore, we have found that supplementation of a culture medium with glutathione also enables cells to grow in a normally inhibitory concentration of 3HP.

Cells incorporating such a genetic modification form an improved platform for further genetic engineering to provide a 3HP expression pathway in the cells.

Summary of the invention

In a first aspect, the invention provides a cell having a metabolic pathway producing 3-hydroxypropionic acid (3HP), said cell exhibiting tolerance for 3HP and having one or more genetic modifications that provide for an enhanced activity of 3HP detoxification by a reaction pathway that includes a glutathione-dependent dehydrogenase reaction.

A said genetic modification conferring said tolerance may be one or more mutations in a gene encoding a glutathione-dependent formaldehyde dehydrogenase. Said one or more mutations may be in a gene equivalent to *SFA1* of *Saccharomyces cerevisiae*, the gene sequence of which is seen in SEQ ID NO 1.

Thus, for instance, said one or more mutations may be in a gene encoding a protein having the sequence SEQ ID NO 1 or a protein with more than 80% homology to SEQ ID NO 1.

The term '3HP detoxification' as used herein includes any process by which a cell is enabled to tolerate the presence of 3-HP at a concentration that would otherwise be detrimental. It is thereby recognised that the toxic effects normally exhibited by 3-HP may be the consequence of 3-HP being transformed *in vivo* to a more directly toxic compound, and detoxification includes reducing the concentration of such a more toxic 3HP metabolite.

The reaction pathway may be one in which S-(3-hydroxypropanoyl_glutathione is converted to 3-HP.

Preferably, the mutation is at a position equivalent to the position aa276 Cys and/or at aa283 Met of the Sfa1p of *Saccharomyces cerevisiae*. Suitable specific mutations include Cys276->Ser, Cys276->Val, Cys276->Thr, Cys276->Gly, Cys276->Ala and/or Met283->Ile, Met283->Ala, Met283->Val. However, other amino acids may be substituted in these positions with like effect.

A said genetic modification may be such as to produce overexpression of a native, or heterologous, or mutated glutathione-dependent formaldehyde dehydrogenase to confer said 3HP tolerance.

Generally, a genetic mutation may be said to produce overexpression of a gene when the production of the gene product of the overexpressed gene is increased as compared to that of a parent organism (which may be termed 'wild type') which lacks said genetic modification but is otherwise the same as the overexpressing organism. Said increase may for instance be by 25% or more, 50% or more, 100% or more, or 200% or more. The genetic modification may involve the introduction of one or more further copies of a gene expressing the relevant gene product. The genetic modification may alternatively or additionally involve the use of a heterologous promoter or a more active endogenous promoter to control expression of a gene expressing the relevant gene product. Forced evolution or mutagenesis may be used to produce micro-organism strains which

overexpress one or more native genes. All such methods may be applied to the overexpression of any gene product referred to herein.

Preferably, said genetic modification produces overexpression of a native or heterologous glutathione-dependent formaldehyde dehydrogenase which has the sequence SEQ ID NO 1 or is a protein with more than 80% homology to SEQ ID NO 1.

Additionally or alternatively, the cell is genetically modified for increased production of glutathione. To this end, it may be that the cell overexpresses the glutathione biosynthetic genes gamma-glutamylcysteine synthetase and glutathione synthetase. Additionally or alternatively, it may be that the cell overexpresses genes that enhance the production of amino acid precursors for glutathione biosynthesis.

3HP producing pathways may comprise several combinations of exogenous genes and overexpressed native genes. In general, 3HP can be obtained by utilizing pathways via at least four different intermediates: malonyl-CoA, glycerol, lactate, or beta-alanine (Figure 1). Bacterial or fungal hosts can be used, where fungal hosts have an advantage of being able to tolerate low pH, therefore enabling a more economical process without the need for neutralization during fermentation and acidification on recovery.

Malonyl-CoA can be reduced to 3HP via combined action of malonyl-CoA reductase (malonate semialdehyde-forming) (E.C. 1.2.1.75) and 3-hydroxypropionate dehydrogenase (E.C. 1.1.1.59/E.C. 1.1.1.298) or 3-hydroxyisobutyrate dehydrogenase (E.C. 1.1.1.31) or by a bi-functional malonyl-CoA reductase (E.C. 1.2.1.75_1.1.1.298), for example from *Chloroflexus aurantiacus* (WO02/42418). Furthermore the host can be engineered for improved supply of precursor and redox co-factor as MCR requires NADPH (Hügler et al., 2002).

Glycerol conversion to 3HP via glycerol dehydratase (E.C. 4.2.1.30) and aldehyde dehydrogenase by engineered *E. coli* and native and engineered *Klebsiella* has been reported most extensively

(Kwak et al., 2012, Rathnasingh, et al., 2009, Luo et al., 2011, Luo et al., 2012, WO2001/016346).

Expression of active enzymes for conversion of lactate into 3HP has been described in WO02/42418, however this route is thermodynamically unfavorable and likely to result in a mixture of lactate and 3HP. Additionally as hydroxymutase, which could convert lactate directly into 3HP, is not found, an alternative 4-step route via propionate CoA-transferase (E.C. 2.8.3.1), lactoyl-CoA dehydratase (E.C. 4.2.1.54), enoyl-CoA hydratase (E.C. 4.2.1.17) and general CoA-lyase (E.C. 6.2.1.-) or 3-hydroxyisobutyryl-CoA hydrolase (E.C. 3.1.2.4) is used.

Finally the route where beta-alanine is converted into malonyl-semialdehyde either by the action of beta-alanine-pyruvate aminotransferase (E.C. 2.6.1.18) or 3-hydroxyisobutyrate dehydrogenase (E.C. 1.1.1.31) has been reported in *E. coli* (US2007/0107080A1). A process utilizing GabT in a non-conventional yeast has been reported (WO2012/074818). Malonyl-semialdehyde is further reduced into 3HP by the action of HIBADH or HPDH. Beta-alanine is present in the cells a product of aspartate degradation or it can be generated from L-alanine by an engineered 2,3-aminomutase with specificity for L-alanine (US2010/0136638A1).

The metabolic pathways producing 3HP may involve several genes, any and all of which may be exogenous or endogenous to the organism to which the cell of the invention belongs.

Thus, said metabolic pathway preferably comprises the enzyme malonyl-CoA reductase and/or the enzyme malonyl-CoA reductase (malonate semialdehyde-forming) in combination with the enzyme 3-hydroxyisobutyrate dehydrogenase and/or the enzyme hydroxypropionate dehydrogenase.

Said metabolic pathway preferably comprises a malonyl-CoA reductase gene, which may be the malonyl-CoA reductase gene from *Chloroflexus aurantiacus* (*CaMCR*), and may comprise an acetyl-CoA

carboxylase gene. Thus, the metabolic pathway may include both the malonyl-CoA reductase gene from *Chloroflexus aurantiacus* (*CaMCR*) and the acetyl-CoA carboxylase gene (*ACC1*) of *S. cerevisiae*.

Optionally said metabolic pathway comprises beta-alanine pyruvate aminotransferase and/or gamma-aminobutyrate transaminase in combination with hydroxyisobutyrate dehydrogenase and/or hydroxypropionate dehydrogenase.

Optionally, said metabolic pathway comprises lactate dehydrogenase, propionate CoA-transferase, lactoyl-CoA dehydratase, enoyl-CoA hydratase and 3-hydroxyisobutyryl-CoA hydrolase.

Optionally, said metabolic pathway comprises glycerol dehydratase and alcohol dehydrogenase.

Optionally, said metabolic pathway comprises the beta-alanine pyruvate aminotransferase from *Bacillus cereus* together with one of the following: 3-hydroxypropanoate dehydrogenase from *Metallosphaera sedula*, 3-hydroxypropanoate dehydrogenase from *Sulfolobus tokadaii*, 3-hydroxypropanoate dehydrogenase from *E. coli* (YdfGp), 3-hydroxypropanoate dehydrogenase from *E. coli* (RutEp), 3-hydroxyisobutyrate dehydrogenase from *Pseudomonas aeruginosa*, 3-hydroxyisobutyrate dehydrogenase from *P. putida*, 3-hydroxyisobutyrate dehydrogenase from *Bacillus cereus*, or 3-hydroxyisobutyrate dehydrogenase from *Candida albicans*.

The cell is preferably a yeast cell, especially a *Saccharomyces cerevisiae cell*. In *Saccharomyces cerevisiae* in particular said genetic modification preferably comprises one or more mutations in gene *SFA1* at aa276 Cys and/or at aa283 Met.

However, the invention is not limited in this regard and the cell may be a fungal cell. Examples of fungal cells include, without limitation, *Aspergillus*, *Fusarium*, *Neurospora*, *Penicillium*, and *Trichoderma* taxonomical classes. Examples of suitable yeast include, without limitation, *Saccharomyces*, *Schizosaccharomyces*, *Kluyveromyces*, *Pichia*, *Candida*, *Yarrowia*, *Brettanomyces*, *Hansenula*, *Lipomyces*, and *Issatchenkia* taxonomical classes.The cell may also be

bacterial rather than fungal and may for instance be of the genus *Eschericia, Lactobacillus, Lactococcus, Corynebacterium, Clostridium,* or *Bacillus.*

The cell may be by way of example S. *cerevisiae, S. pombe, S. kluyveri, K. lactis, K. marxianus, Y. lipolytica, T. delbreueckii, R. minuta, I. orientalis, P. stipites, L. starkeyi, C. guilliermondii,* or *E. coli.*

In a further aspect, the invention provides a yeast exhibiting tolerance for 3HP, said yeast having a mutation in gene *SFA1* conferring said tolerance.

Optionally, the yeast is *Saccharomyces cerevisiae* and and said mutation in gene *SFA1* is at aa276 Cys and/or at aa283 Met. said mutation in gene *SFA1* is Cys276->Ser, Cys276->Val, Cys276->Thr, Cys276->Gly, Cys276->Ala and/or Met276->Ile, Met276->Ala, Met276->Val. In particular, it is preferred that said mutation in gene *SFA1* is Cys->Ser(aa276) and/or Met->Ile(aa283).

In a further aspect, the invention includes a method of producing 3HP comprising cultivating a 3HP producing cell under 3HP producing conditions in a culture medium so as to produce 3HP, wherein toxicity of 3HP is reduced by an enhanced activity of 3HP detoxification by a reaction pathway that includes a glutathione-dependent dehydrogenase reaction.

The enhanced activity of said pathway may be provided by genetic modification as described above but additionally or alternatively may be provided by said culture medium being supplemented with glutathione and/or cysteine, glutamate, glycine, serine, methionine, and arginine (Wen et al., 2004).

Preferably, glutathione is added to the culture medium to produce a concentration therein of >2.5 mM.

Instead or additional to addition of glutathione, the cell may have a genetic modification conferring an enhanced glutathione production ability thereon. This may be such that the cell

overexpresses the glutathione biosynthetic genes gamma-glutamylcysteine synthetase and glutathione synthetase. Alternatively or additionally it may be such that the cell overexpresses genes that enhance the production of amino acid precursors for glutathione biosynthesis.

In view of the enhanced 3HP tolerance of the cells, the concentration of 3HP in the culture medium may be permitted to rise to in excess of 1 g/l, e.g. in excess of 3, or 4 or 5 g/l or 10 g/l.

In a further aspect, the invention provides the use of an enhanced glutathione-dependent dehydrogenase reaction in a cell to enhance tolerance of said cell to 3HP.

Optionally, reaction is one converting S-(3-hydroxypropanoyl)glutathione to S-(3-ketopropanoyl)glutathione.

Description of the drawings

The invention will be further described and illustrated with reference to the accompanying drawings in which:

Figure 1 illustrates pathways leading from pyruvate to 3HP; The following abbreviations are used:

PYC – pyruvate carboxylase,

AAT – aspartate aminotransferase,

PanD – aspartate decarboxylase,

BAPAT – beta-alanine pyruvate aminotransferase,

GabT – gamma-aminobutyrate transaminase,

HIBADH – 3-hydroxyisobutyrate dehydrogenase,

HPDH – hydroxypropionate dehydrogenase,

HPDH - 3-hydroxypropionate dehydrogenase,

LDH – lactate dehydrogenase,

PCT - propionate CoA-transferase,

LCD - lactoyl-CoA dehydratase,

ECH - enoyl-CoA hydratase,

HIBCH - 3-hydroxyisobutyryl-CoA hydrolase.

MCR1 - malonyl-CoA reductase (malonate semialdehyde-forming),

MCR2 - bi-functional malonyl-CoA reductase,

GD – glycerol dehydratase,

ALD – aldehyde dehydrogenase

Figure 2 shows growth curves for *S. cerevisiae* on minimal medium in the presence of different concentrations of 3HP, as described in Example 1;

Figure 3 shows improved 3HP tolerance of strains of *S. cerevisiae* having overexpressed native or mutated *SFA1* genes as found in Example 2;

Figure 4 shows improved 3HP tolerance of strains of *S. cerevisiae* having mutated *SFA1* genes at varying levels of 3HP in the minimal medium, as found in Example 3;

Figure 5 shows 3HP titers and yields in strains having 3HP pathway genes in combination with or without having overexpressed native or mutant *SFA1* genes, as found in Example 4;

Figure 6 shows the proposed 3HP detoxification pathway in *S. cerevisiae*;

Figure 7 shows the effect of GSH on the growth of strains of *S. cerevisiae* having the native or mutated *SFA1* gene as well as the *sfa1Δ* strains grown on minimal medium containing 50 g/L 3HP, as described in Example 5;

Figure 8 shows the growth of SFA1-alleles replacement strains grown on minimal medium containing 50 g/L 3HP, as described in Example 8;

Figure 9 shows the effect of GSH addition on the growth of several yeast strains grown on minimal medium containing 50 g/L 3HP, as described in Example 9;

Figure 10 shows shows the effect of GSH addition on the growth of several *E. coli* strains grown on M9 containing 20 g/L 3HP, as described in Example 9.

Examples

The following examples demonstrate the effectiveness of the invention. The following tables summarise materials used in the Examples and results obtained.

Table 1. Primers

Primer name	Primer sequence, 5'->3'	SEQ ID NO
KO_sfa1_fw	CAGAATTTGTTGGCCTATTTTCTTA	SEQ ID NO 16
KO_sfa1_rv	CAATACGTTGGTAGTTAGGAACAGG	SEQ ID NO 17
KO_sfa1_test_fw	GATGCTCATCACAGACTACT	SEQ ID NO 18
KanMX_2/3_START_rv	AGTGACGACTGAATCCGGTG	SEQ ID NO 19
PTEF1_fw	ACCTGCACUTTGTAATTAAAACTTAG	SEQ ID NO 20
PTEF1_rv	CACGCGAUGCACACACCATAGCTTC	SEQ ID NO 21
SFA1_U1_fw	AGTGCAGGUAAAACAATGTCCGCCGCTACTGTT	SEQ ID NO 22
SFA1_U1_rv	CGTGCGAUTCATTTATTTCATCAGACTTCAAGA	SEQ ID NO 23
SFA1_UPrevU1	AGCTGTTCUCTATTTTATTTCATCAGACTTCAAGACG	SEQ ID NO 24
SFA1_DWfwd_U2	AGTGGCCUGAGTACTTAATTAAACTAAGTAAGCATGACTC	SEQ ID NO 25
KO-SFA1_UPrev2	GTCTACCGTGATTTCTTCAACACTT	SEQ ID NO 26
NB336K1LEUFwd1	TGGAAGAGGCAAGCACGTTAGC	SEQ ID NO 27

NB335K1LEURev1	CAGAAGCATAACTACCCATTCC	SEQ ID NO 28
NB326URA3fwdU	AGAACAGCUGAAGCTTCGTACG	SEQ ID NO 29
NB327URA3Rev2U	AGGCCACUAGTGGATCTGATATCAC	SEQ ID NO 30
PPGK1_rv	CACGCGAUGCACACACCATAGCTTC	SEQ ID NO 31
gsh1_U1_fw	AGTGCAGGUAAAACAATGGGACTCTTAGCTTTGGG	SEQ ID NO 32
gsh1_U1_rv	CGTGCGAUTCAACATTTGCTTTCTATTGAAGGC	SEQ ID NO 33
gsh2_U1_fw	ATCTGTCAUAAAACAATGGCACACTATCCACCTTCC	SEQ ID NO 34
gsh2_U1_rv	CACGCGAUTCAGTAAAGAATAATACTGTCC	SEQ ID NO 35
met14_U1_fw	AGTGCAGGUAAAACAATGGCTACTAATATTACTTGGC	SEQ ID NO 36
met14_U1_rv	CGTGCGAUTCACAAATGCTTACGGATGATTTTTTC	SEQ ID NO 37
met16_U1_fw	ATCTGTCAUAAAACAATGAAGACCTATCATTTG	SEQ ID NO 38
met16_U1_ev	CACGCGAUTCAGGCATCTTGCTTTAAAAATTGC	SEQ ID NO 39
SFA1_G614C_fw	ATACCGTTGCAGTATTTGGCTCCGGGACTGTAG	SEQ ID NO 40
SFA1_G614C_rv	CTACAGTCCCGGAGCCAAATACTGCAACGGTAT	SEQ ID NO 41
SFA1_G710C_fw	GCCATTGACATTAACAATAAGAAAAAACAATATTCTTCTCAATTTGGTGCCAC	SEQ ID NO 42

SFA1_G710C_rv	GTGGCACCAAATTGAGAAGAATATTGTTTTTTCTTATTGTTAATGTCAATGGC	SEQ ID NO 43
SFA1_G869C_fw	GAGAGATGCTTTGGAAGCCTCTCATAAAGGTTGGG	SEQ ID NO 44
SFA1_G869C_rv	CCCAACCTTTATGAGAGGCTTCCAAAGCATCTCTC	SEQ ID NO 45
SFA1_T826G_fw	GGGGTCTGGATTTTACTTTTGACGGTACTGGTAATACCAAAATTATG	SEQ ID NO 46
SFA1_T826G_rv	CATAATTTTGGTATTACCAGTACCGTCAAAAGTAAAATCCAGACCCC	SEQ ID NO 47
SFA1_TG826GC_fw	GGGGTCTGGATTTTACTTTTGACGCTACTGGTAATACCAAAATTATG	SEQ ID NO 48
SFA1_TG826GC_rv	CATAATTTTGGTATTACCAGTAGCGTCAAAAGTAAAATCCAGACCCC	SEQ ID NO 49
SFA1_TG826GT_fw	GGGGGTCTGGATTTTACTTTTGACGTTACTGGTAATACCAAAATTATGAG	SEQ ID NO 50
SFA1_TG826GT_rv	CTCATAATTTTGGTATTACCAGTAACGTCAAAAGTAAAATCCAGACCCCC	SEQ ID NO 51
SFA1_T826A_fw	GGGGTCTGGATTTTACTTTTGACACTACTGGTAATACCAAAATTATG	SEQ ID NO 52
SFA1_T826A_rv	CATAATTTTGGTATTACCAGTAGTGTCAAAAGTAAAATCCAGACCCC	SEQ ID NO 53
SFA1_A847G_fw	CTTTTGACTGTACTGGTAATACCAAAATTGTGAGAGATGCTTTGG	SEQ ID NO 54
SFA1_A847G_rv	CCAAAGCATCTCTCACAATTTTGGTATTACCAGTACAGTCAAAAG	SEQ ID NO 55
SFA1_AT847GC_fw	TTACTTTTGACTGTACTGGTAATACCAAAATTGCGAGAGATGCTTTGGAAG	SEQ ID NO 56
SFA1_AT847GC_rv	CTTCCAAAGCATCTCTCGCAATTTTGGTATTACCAGTACAGTCAAAAGTAA	SEQ ID NO 57

Table 2. Primers and templates used to generate gene fragments for gene reaplcement

Fragment name	**Gene**	**Fw_primer**	**Rv_primer**	**Template DNA**
SFA1_UP	Upstream region of SFA1	KO_sfa1_fw	KO-SFA1_UPrev2	gDNA of *S. cerevisiae*
SFA1wt_UP	Upstream region of SFA1 including SFA1-wt	KO_sfa1_fw	SFA1_UPrevU1	gDNA of *S. cerevisiae*
$SFA1^{G827C}$_UP	SFA1 upstream region fused by PCR to the $SFA1^{G827C}$ gene	KO_sfa1_fw	SFA1_UPrevU1	Fragment SFA1_UP_wt and $SFA1^{G827C}$
$SFA1G^{849A}$_UP	SFA1 upstream region fused by PCR to the $SFA1^{G849A}$ gene	KO_sfa1_fw	SFA1_UPrevU1	Fragment SFA1_UP_wt and $SFA1^{G849A}$
SFA1_DOWN	Downstream region of SFA1	SFA1_DWfwd_U2	SFA1_DW_test_rv	gDNA of *S. cerevisiae*
LEU2_U_2/3_START	the first 2/3 of loxP-KlLEU2 marker	NB326URA3fwdU	NB335KlLEURev1	Plasmid pUG73
LEU2_U_2/3_END	the last 2/3 part of loxP-KlLEU2 marker	NB336KlLEUFwd1	NB327URA3Rev2U	Plasmid pUG73
SFA1wt_UP_LEU2_U_2/3_START	SFA1 upstream region including SFA1-wt fused by PCR to the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP_wt and LEU2_U_2/3_START
$SFA1^{G827C}$_UP_LEU2_U_2/3_START	SFA1 upstream region including $SFA1^{G827C}$ fused by PCR to the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment $SFA1^{G827C}$_UP and LEU2_U_2/3_START

$SFA1^{G849A}$_UP_LEU2_U_2/3_START	SFA1 upstream region including $SFA1^{G849A}$ fused by PCR to the first 2/3 of K1LEU2 marker	KO_sfa1_fw	NB335K1LEURev1	Fragment $SFA1^{G849A}$_UP and LEU2_U_2/3_START
LEU2_U_2/3_END_SFA1_DOWN	the last part of K1LEU2 marker fused by PCR to SFA1 downstream region	NB336K1LEUFwd1	SFA1_DW_test_rv	Fragment LEU2_U_2/3_END and SFA1_DOWN
$SFA1^{G614C}$	*SFA1* gene where nt 614 was changed from G to C (Cys205Ser)	SFA1_U1_fw	SFA1_U1_rv	pESC-LEU-$SFA1^{G614C}$
$SFA1^{G710C}$	*SFA1* gene where nt 710 was changed from G to C (Cys237Ser)	SFA1_U1_fw	SFA1_U1_rv	pESC-LEU-$SFA1^{G710C}$
$SFA1^{G869C}$	*SFA1* gene where nt 869 was changed from G to C (Cys290Ser)	SFA1_U1_fw	SFA1_U1_rv	pESC-LEU-$SFA1^{G869C}$
$SFA1^{T826G}$	*SFA1* gene where nt 826 was changed from T to G (Cys276Gly)	SFA1_U1_fw	SFA1_U1_rv	pESC-LEU-$SFA1^{T826G}$
$SFA1^{TG826GC}$	*SFA1* gene where nt 826 and 827 wer changed from T to G and G to C, respectively (Cys276Ala)	SFA1_U1_fw	SFA1_U1_rv	pESC-LEU-$SFA1^{TG826GC}$
$SFA1^{TG826GT}$	*SFA1* gene where nt 826 and 827 wer changed from T to G and G to T, respectively (Cys276Val)	SFA1_U1_fw	SFA1_U1_rv	pESC-LEU-$SFA1^{TG826GT}$

SFA1^{TG826AC}	*SFA1* gene where nt 826 and 827 wer changed from T to A and G to C, respectively (Cys276Thr)	SFA1_U1_fw	SFA1_U1_rv	pESC-LEU-SFA1^{TG826AC}
SFA1^{A847G}	*SFA1* gene where nt 847 was changed from A to G (Met283Val)	SFA1_U1_fw	SFA1_U1_rv	pESC-LEU-SFA1^{A847G}
SFA1^{AT847GC}	*SFA1* gene where nt 847 and 848 wer changed from A to G and T to C, respectively (Met283Ala)	SFA1_U1_fw	SFA1_U1_rv	pESC-LEU-SFA1^{AT847GC}
SFA1^{G614C}_UP_LEU2_U_2/3_START	SFA1 upstream region fused by PCR to the including SFA1^{G614C} gene and the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP, SFA1^{G614C} and LEU2_U_2/3_START
SFA1^{G710C}_UP_LEU2_U_2/3_START	SFA1 upstream region fused by PCR to the including SFA1^{G710C} gene and the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP, SFA1^{G710C} and LEU2_U_2/3_START
SFA1^{G869C}_UP_LEU2_U_2/3_START	SFA1 upstream region fused by PCR to the including SFA1^{G869C} gene and the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP, SFA1^{G869C} and LEU2_U_2/3_START
SFA1^{T826C}_UP_LEU2_U_2/3_START	SFA1 upstream region fused by PCR to the including SFA1^{T826C} gene and the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP, SFA1^{T826C} and LEU2_U_2/3_START

SFA1^{TG826GC}_UP_LEU2_U_2/3_START	SFA1 upstream region fused by PCR to the including SFA1^{TG826GC} gene and the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP, SFA1^{TG826GC} and LEU2_U_2/3_START
SFA1^{TG826GT}_UP_LEU2_U_2/3_START	SFA1 upstream region fused by PCR to the including SFA1^{TG826GT} gene and the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP, SFA1^{TG826GT} and LEU2_U_2/3_START
SFA1^{TG826AC}_UP_LEU2_U_2/3_START	SFA1 upstream region fused by PCR to the including SFA1^{TG826AC} gene and the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP, SFA1^{TG826AC} and LEU2_U_2/3_START
SFA1^{A847C}_UP_LEU2_U_2/3_START	SFA1 upstream region fused by PCR to the including SFA1^{A847C} gene and the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP, SFA1^{A847C} and LEU2_U_2/3_START
SFA1^{AT847GC}_UP_LEU2_U_2/3_START	SFA1 upstream region fused by PCR to the including SFA1^{AT847G} gene and the first 2/3 of KlLEU2 marker	KO_sfa1_fw	NB335KlLEURev1	Fragment SFA1_UP, SFA1^{AT847GC} and LEU2_U_2/3_START

Table 3. Primers and templates used to generate gene fragments for USER cloning by PCR

Fragment name	**Gene**	**Fw_primer**	**Rv_primer**	**Template DNA**
PTEF1	Promoter of *TEF1* gene from *S. cerevisiae*	PTEF1_fw	PTEF1_rv	gDNA of *S.*

SFA1-wt	SFA1 gene (WT sequence)	SFA1_U1_fw	SFA1_U1_rv	gDNA of *S. cerevisiae*
SFA1^{G827C}	SFA1 gene where nt 827 was changed from G to C	SFA1_U1_fw	SFA1_U1_rv	gDNA of *S. cerevisiae*
SFA1^{G849A}	SFA1 gene where nt 849 was changed from G to A	SFA1_U1_fw	SFA1_U1_rv	gDNA of *S. cerevisiae*
<-PTEF1-PPGK1->	Fused promoters of *TEF1* and *PGK1* genes from *S. cerevisiae*	PTEF1_fw	PPGK1_rv	plasmid pSP-GM1
GSH1<-	Gamma glutamylcysteine synthetase (GSH1) from *S. cerevisiae*	gsh1_U1_fw	gsh2_U1_rv	gDNA of *S. cerevisiae*
GSH2->	Glutathione synthetase (*GSH2*) from *S. cerevisiae*	gsh1_U1_fw	gsh2_U1_rv	gDNA of *S. cerevisiae*
MET14<-	Adynylylsulfate kinase (*MET14*) from *S. cerevisiae*	gsh1_U1_fw	met14_U1_rv	gDNA of *S. cerevisiae*
MET16->	3'-phosphoadenylsulfate reductase (*MET16*) from *S. cerevisiae*	met16_U1-fw	met16_U1-rv	gDNA of *S. cerevisiae*

Table 4. Plasmids

Plasmid name	Parent plasmid	Selection marker	Cloned fragment	Promoter	Terminator
pESC-LEU		LEU2	-	-	-
pESC-LEU-USER		LEU2	-	-	-
pUG73		K1LEU2	-	-	-
pESC-LEU-SFA1-wt	pESC-LEU-USER	LEU2	SFA1-wt	PTEF1	TADH1
pESC-LEU-SFA1^{G827C}	pESC-LEU-USER	LEU2	SFA1^{G827C}	PTEF1	TADH1
pESC-LEU-SFA1^{G849A}	pESC-LEU-USER	LEU2	SFA1^{G849A}	PTEF1	TADH1
pESC-URA	-				
pESC-URA-USER	-				
pESC-URA-GSH1-GSH2	pESC-URA-USER				
pESC-HIS	-				
pESC-HIS-USER	-				
pESC-HIS-MET14-MET16	pESC-HIS-USER				
pESC-LEU-SFA1^{G614C}	pESC-LEU-USER				
pESC-LEU-SFA1^{G710C}	pESC-LEU-USER				

pESC-LEU-SFA1^{G869C}	pESC-LEU-USER				
pESC-LEU-SFA1^{T826G}	pESC-LEU-USER				
pESC-LEU-SFA1^{TG826GC}	pESC-LEU-USER				
pESC-LEU-SFA1^{TG826GT}	pESC-LEU-USER				
pESC-LEU-SFA1^{T826A}	pESC-LEU-USER				
pESC-LEU-SFA1^{A847G}	pESC-LEU-USER				
pESC-LEU-SFA1^{AT847GC}	pESC-LEU-USER				

Table 5. Yeast strains

Strain name	**Genotype**
CEN.PK 113-7D	*MATa URA3 HIS3 LEU2 TRP1*
CEN.PK 113-32D	*MATa leu2Δ*
CEN.PK 102-5B	*MATa ura3Δ leu2Δ his3Δ*
sfa1Δ	*MATa leu2Δ sfa1::KanMX*
SCE-R1-48	*MATa leu2Δ sfa1::KanMX* + pESC-LEU
SCE-R1-49	*MATa leu2Δ sfa1::KanMX* + pESC-LEU-SFA1-wt
SCE-R1-50	*MATa leu2Δ sfa1::KanMX* + pESC-LEU-SFA1 ^{G827C}
SCE-R1-51	*MATa leu2Δ sfa1::KanMX* + pESC-LEU-SFA1 ^{G849A}
ST609	*MATa leu2Δ sfa1::SFA1-wt:KlLEU2*
ST610	*MATa leu2Δ sfa1::SFA1^{G827C}:KlLEU2*
ST611	*MATa leu2Δ sfa1::SFA1^{G849A}:KlLEU2*
ST637	*MATa leu2Δ ACC1-CaMCR:KlURA3 ACSse-ALD6:KlLEU2 PDC1:SpHIS5*
ST726	*MATa ura3Δ leu2Δ his3Δ* sfa1:: *SFA1^{G827C}:KlLEU2*

ST727	*MATa ura3Δ leu2Δ his3Δ* sfa1:: *SFA1^{G849A}:K1LEU2*
ST728	*MATa ura3Δ leu2Δ his3Δ* sfa1:: *SFA1wt:K1LEU2*

Table 6. Strains and 3HP titers in strains producing 3HP via malonyl-CoA pathway

Strain name	**Parent strain**	**Plasmid with LEU2 marker**	**3HP titer (g/L) in Delft media**	**3HP titer (g/L) in FIT media**
wt	**CEN.PK113-7D**	**-**	0.1±0.01	0.4±0.02
R2-129	**ST637**	**pESC-LEU**	1.8±0.4	5.4±0.9
R2-130	**ST637**	**pESC-LEU-SFA1-wt**	2.1±0.4	5.9±1.0
R2-131	**ST637**	**pESC-LEU-SFA1^{G827C}**	1.8±0.4	5.7±0.5
R2-132	**ST637**	**pESC-LEU-SFA1^{G849A}**	2.0±0.2	5.8±0.9

Example 1. Toxicity of 3-hydroxypropionic acid (3HP)

The toxicity of 3HP on growth of *S. cerevisiae* CEN.PK 113-7D strain(WT) was determined by evaluating the ability of the WT strain to grow on a chemically defined minimal medium (Delft) containing varying concentrations of 3HP (0 - 100 g/L). The composition of Delft medium was as follows: 20.0 g/L glucose, 5.0 g/L $(NH_4)_2SO_4$, 3.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 1.0 mL/L trace metal solution, 0.05 g/L antifoam A (10794, Sigma-Aldrich), and 1.0 mL/L vitamin solution. The trace element solution included 15 g/L EDTA, 0.45 g/L $CaCl_2.2H_2O$, 0.45 g/L $ZnSO_4$ $.7H_2O$, 0.3 g/L $FeSO_4.7H_2O$, 100 mg/L H_3BO_4, 1 g/L $MnCl_2.2H_2O$,0.3 g/L $CoCl_2.6H_2O$, 0.3 g/L $CuSO_4.5H_2O$, 0.4 g/L $NaMoO_4.2H_2O$. The pH of the trace metal solution was adjusted to 4.0 with 2 M NaOH and heat sterilized. The vitamin solution included 50 mg/L d-biotin, 200 mg/L para-amino benzoic acid, 1 g/L nicotinic acid, 1 g/L Ca.pantothenate, 1 g/L pyridoxine HCl, 1 g/L thiamine HCl, and 25 mg/L m. inositol. The pH of the vitamin solution was adjusted to 6.5 with 2 M NaOH, sterile-filtered and the solution was stored at 4°C. The Delft medium was prepared as a concentrate solution and the pH was adjusted to 6.0 and sterilized by autoclaving. Glucose was autoclaved separately. The final Delft medium (Delft buffered) was obtained by adding 79.5 mL of 0.5 M citrate solution (105 g/L $C_6H_8O_7.H_2O$) and 20.5 mL of 1M sodium phosphate solution (178 g/L $Na_2HPO_4.2H_2O$) to control the pH of the medium at pH 3.5. For media containing 3HP, the 3HP solution (ca. 30% in water; Tokyo Chemical Industry Co.) was neutralized with solid NaOH (0.133 g NaOH/1 mL of 3HP solution) and sterile-filtered before adding to the Delft buffered media.

A single colony from YPD plate (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar) was inoculated in a shake flask with 20 mL YPD broth and grown at 30°C, with shaking at 200 rpm, overnight. The absorbance (OD_{600}) of the overnight culture was measured at 600 nm wave length using NanoPhotometer™ UV/Vis (Implen) and the culture was diluted with YPD medium to obtain inoculum with the OD_{600} around 0.8. The diluted culture was used to inoculate 100 µL Delft buffered medium (pH 3.5) containing various concentration of 3HP (0-100 g/L) in 96-well flat bottom plate (Greiner) with the

starting inoculum of OD_{600} = 0.04. The 96-well plate was incubated at 30°C with shaking in the Synergy™ MX microplate reader (BioTek) and the absorbance was measured at 600 nm wavelength every 15 min for 42 hours. Experiments were done in duplicates, and the specific growth rate (h^{-1}) was calculated at various concentrations of 3HP. The resulting of 3HP sensitivity is shown in Figure 2.

It can be seen that any concentration of 3-HP much above 10 g/L produces a substantial level of inhibition and the wild type yeast is unable to grow at all in concentrations of 75 g/L.

Example 2. Metabolic engineering for improving 3HP tolerance in *S. cerevisae*

A strain with *SFA1* deletion was constructed and tested for 3HP tolerance. In addition, three versions of the *SFA1* gene (native and two alleles) were overexpressed in the *sfa1Δ* and WT strains and 3HP tolerance was investigated. Deletion of *SFA1* did not result in any 3HP tolerance. However, overexpression of *SFA1* gene (native and two alleles) enabled growth on 50 g/L 3HP of the WT and *sfa1Δ* strains. The two alleles are referred to herein as $SFA1^{G827C}$ (Cys→Ser(aa276)) and $SFA1^{G849A}$ (Met→Ile(aa283)).

The *sfa1Δ* strain was constructed by replacing the target gene in the CEN.PK113-32D strain with the *KanMX* cassette.

The gene fragments carrying the *KanMX cassette* and correct overhangs for *SFA1* replacement (KO-SFA1) was generated by PCR amplification using primers and template as indicated in Table 2. The PCR mix contained: 31 µl water, 10 µl high fidelity Phusion® polymerase buffer (5x), 5 µl 2mM dNTP, 1 µl Phusion® polymerase, 1 µl forward primer at 10 µM concentration, 1 µl reverse primer at 10 µM concentration, and 1 µl DNA template. The cycling program was: 95°C for 2 min, 30 cycles of [95°C for 10 sec, 50°C for 20 sec, 68°C for 1 min], 68°C for 5 min, pause at 10°C. The gene fragments were resolved on 1% agarose gel containing SYBR®-SAFE (Invitrogen) and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel).

The KO-SFA1 fragment was transformed into *S. cerevisiae* cells using the lithium acetate transformation protocol. The cells were selected on YPD plate with G418 (200 µg/mL). The transformants were streak purified on YPD+G418 plate in order to obtain single colonies. The correct transformants were confirmed by PCR analysis using primers KO_sfa1_test_fw/KanMX_2/3_START_rv.

To construct the overexpression plasmid containing *SFA1* gene, the *SFA1-wt* (SEQ ID NO 1), $SFA1^{G827C}$ (SEQ ID NO 2), $SFA1^{G849A}$ (SEQ ID NO 3) genes were subcloned into pESC-LEU-USER plasmid by USER cloning. The final plasmids (Table 4) were then transformed into different background strains (WT and *sfa1Δ*) using the lithium acetate transformation protocol and selected on synthetic complete agar medium without leucine (SC-Leu). Transformants were streaked purified on SC-Leu to obtain single colonies. The resulting strains are listed in Table 5.

The promoter fragment (PTEF1, SEQ ID 4) was generated by PCR followed by gene purification (Table 3). The terminators were already present on the expression plasmids.

The parent plasmid pESC-LEU-USER were linearized with FastDigest® AsiSI (Fermentas) for 1 hour at 37°C and nicked with Nb.BsmI for 1 hour at 37°C. The resulting linearized nicked DNA was purified from the solution and eluted in 5mM Tris buffer, pH 8.0.

The expression plasmids were created by USER-cloning using the following protocol. One µl of linearized and nicked parent plasmid was mixed with 1 µL of promoter fragment, 2 µL of gene fragment, 0.5 µL Taq polymerase buffer, 0.5 µL USER enzyme (NEB). The mix was incubated at 37°C for 25 min, at 25°C for 25 min and transformed into chemically competent *E. coli* DH5alpha. The clones with correct inserts were identified by colony PCR and the plasmids were isolated from overnight *E. coli* cultures and confirmed by sequencing. The expression plasmids are listed in Table 4. For testing 3HP tolerance phenotype, four single colonies from each strain line were investigated for the ability to grow on media containing 3HP. The pre-cultures were prepared by inoculation a single colony in 100 µL SC-Leu media in 96-well flat bottom plate. The plate was incubated at 30°C with 250 rpm agitation at 5 cm orbit cast overnight. Five

µL of the overnight cultures were used to inoculate 100 µL Delft buffered (pH 3.5) containing 50 g/L 3HP. The 96-well plate was incubated at 30°C with shaking in the Synergy™ MX microplate reader (BioTek) and the absorbance was measured at 600 nm wavelength every 15 min for 42 hours. Experiments were done in triplicate. The resulting improvement in 3HP tolerance in the engineered strains is shown in Figure 3.

In Figure 3, SCE-R1-48 is the *sfa1Δ* strain carrying the empty plasmid pESC-LEU. SCE-R1-49, SCE-R1-50, and SCE-R1-51 are the strains overexpressing *SFA1* in its wild type form, $SFA1^{G827C}$, and $SFA1^{G849A}$, respectively. As seen in Figure 3, all three strains overexpressing a form of *SFA1* have improved 3HP tolerance compared to the *sfa1Δ* strain.

Example 3. Improving 3HP tolerance in *S. cerevisiae* by replacing the native SFA1 with either $SFA1^{G827C}$ or $SFA1^{G849A}$

To investigate whether only the *SFA1* alleles can confer 3HP tolerance without overexpression, the native *SFA1* gene was replaced by either $SFA1^{G827C}$ or $SFA1^{G849A}$ and tested for 3HP tolerance. By replacing the *SFA1-wt* with $SFA1^{G827C}$ or $SFA1^{G849A}$, the constructed strains (ST610 and ST611) could grow on minimum media containing 3HP above 40 g/L, whereas the strain with the *SFA1-wt* allele without over expression (ST609) could not grow under these conditions. The results clearly showed that only one amino acid changed in *SFA1* is enough for *S. cerevisiae* to confer 3HP tolerance.

To generate the substrates for *SFA1* allele replacement, the upstream fragment including the *SFA1* allele for each SFA1 mutations was generated by fusion PCR using the SFA1 upstream region (SFA1-UP), SFA1-allele fragment and the first part of 2/3 KlLEU2 marker as templates and using primers KO_sfa1_fw and NB335KlLEURev1 (Table 2). The downstream fragment was generated by fusion PCR using the last part of 2/3 KlLEU2 marker and the SFA1 downstream region (SFA1_DOWN) as templates and using primers NB336KlLEUFwd1 and SFA1_DW_test_rv. The cycling program was: 98°C for 2 min, 30 cycles of [98°C for 10 sec, 55°C for 30 sec, 68°C for 1 min 30 sec], 68°C for 12 min, pause at 10°C.

The strain with *SFA1*^{G827C} allele replacement was constructed by replacing the *KanMX* cassette in the *sfa1Δ* strain using SFA1^{G827C}_UP_ LEU2_U_2/3_START and LEU2_U_2/3_END_SFA1_DOWN_wt fragments, whereas the *SFA1*^{G849A} strain was constructed in the same manner using SFA1^{G849A}_UP_ LEU2_U_2/3_START and LEU2_U_2/3_END_SFA1_DOWN_wt fragments. For the control strain, the SFA1-wt allele was introduced back to the *sfa1Δ* strain by using SFA1wt_UP_ LEU2_U_2/3_START and LEU2_U_2/3_END_SFA1_DOWN_wt fragments. The strains were selected on SC-Leu media. The transformants were streaked purified on SC-Leu to obtain single colonies. The correct transformants were confirmed by PCR analysis using primers KO_sfa1_test_fw and SFA1_U1-rv.

For testing 3HP tolerance phenotype, two single colonies from each strain: ST609 (SFA1-wt), ST610 (SFA1^{G827C}) and ST611 (SFA1^{G849A}) were investigated for the ability to grow on media containing 3HP. The pre-cultures were prepared by inoculation of a single colony in 0.5 mL Delft buffered (pH 3.5) media in 24-well plate. The plate was incubated at 30°C with 250 rpm agitation at 5 cm orbit cast overnight. Five µL of the overnight cultures were used to inoculate 100 µL Delft buffered (pH 3.5) containing various concentration of 3HP (0, 10, 25, 40 and 50 g/L) in 96-well flat bottom plate. The 96-well plate was incubated at 30°C with shaking in the Synergy™ MX microplate reader (BioTek) and the absorbance was measured at 600 nm wavelength every 15 min for 42 hours. Experiments were done in duplicates. The resulting of 3HP tolerance in the engineered strains is shown in Figure 4.

Example 4. Production of 3-hydroxypropionic acid via malonyl-CoA pathway

To test the influence of 3HP tolerance gene on 3HP production, three versions of the *SFA1* gene (native and two alleles) were overexpressed in the 3HP high producer (ST637) strain and characterized for 3HP production. The resulting strains and 3HP production are shown in Table 6 and Figure 5. The 3HP titers and yields were similar to the or better than the reference strain (R2-

129), showing that they were compatible and even advantageous for the production of 3HP via malonyl-CoA pathway.

The *S. cerevisiae*-3HP high producer strain has been engineered to carry several genetic modifications. Extra copies of the native acetyl-CoA synthetase (*ACSse*; SEQ ID NO 6), aldehyde dehydrogenase (*ALD6*; SEQ ID NO 7), indolepyruvate decarboxylase (*PDC1*; SEQ ID NO 8) and acetyl-CoA carboxylase (*ACC1*; SEQ ID NO 9) were introduced into the CEN.PK102-5B strain to improve the supply of precursor and redox co-factor. These genes were integrated into the genome and were under the control of either PTEF1 (SEQ ID NO 4) or PPGK1 (SEQ ID NO 5) promoters. Furthermore, the malonyl-CoA reductase gene from *Chloroflexus aurantiacus* (*CaMCR*; SEQ ID NO 10) responsible for converting malonyl-CoA into 3HP was also introduced into this strain.

The plasmids conferring 3HP tolerance were transformed into 3HP high producer strain the lithium acetate transformation protocol. The cells were selected on SC-Leu. For the control experiments, the strains were transformed with an empty plasmid pESC-LEU.

Six single colonies originating from four independent transformants were inoculated in 0.5 mL SC-Ura-His-Leu in 96-deep well microtiter plate with air-penetrable lid (EnzyScreen). The plates were incubated at 30°C with 250 rpm agitation at 5 cm orbit cast overnight. 50 µL of the overnight cultures were used to inoculate 0.5 mL Delft or Feed-In-Time (FIT) Fed-batch medium (m2p-labs GmBH) in 96-deep well plate. Cultivation was carried out for 72 hours at the same conditions as above.

At the end of the cultivation the OD_{600} was measured. The cultivation was diluted 20 times in a total volume of 200 µL and absorbance was measured at 600 nm wavelength on spectrophotometer (Synergy™ MX Microplate reader, BioTek).

The culture broth was spun down and the supernatant analyzed for 3HP concentration using enzymatic assay (Table5). Enzymatic assay was carried out as following. 20 µL of standards (3HP at concentrations from 0.03 to 2 g/L in Delft medium) and samples were added to 96-well flat bottom transparent plate (Greiner). 180 µl of

mix (14.8 mL water, 2 mL buffer (1 mM Tris, 25 mM $MgCl_2$, pH 8.8), 1 mL NADP+ solution (50 mg/mL), and 0.2 mL purified YdfG enzyme in PBS buffer (1500 µg/mL)) was added per well using multichannel pipet. The start absorbance at 340 nm was measured; the plate was sealed and incubated at 30°C for 1.5 hours. After that the end absorbance at 340 nm was measured again. The difference between the end and the start values corrected for the background were in linear correlation with 3HP concentrations. The concentration of 3HP in the samples was calculated from the standard curve.

Example 5. The proposed 3HP detoxification pathway.

The proposed function of SFA1 protein (Sfa1p) in formaldehyde detoxification pathway has been reported (Yasokawa *et al.*, 2010). Formaldehyde is spontaneously reacted with intracellular glutathione (GSH) to form S-hydroxymethylglutathione which is then converted into S-formylglutathione by the Sfa1p and required $NAD(P)^+$ as a cofactor. We proposed that 3HP detoxification by the SFA1p might be similar to that observed in formaldehyde detoxification and GSH might also be involved in this process. The proposed 3HP detoxification pathway is shown in Figure 6.

In vivo S. cerevisiae strain can convert 3HP into 3-hydroxypropionaldehyde (3HPA) by the aldehyde dehydrogenases (ALDs). As 3HPA is much more toxic than 3HP, yeast cells must efficiently eliminate this lethal compound by converting it into other less toxic compounds. 3HPA can spontaneously bind to glutathione to form S-(3-hydroxypropanoyl)glutathione which is then oxidized into S-(3-ketopropanoyl)glutathione by the Sfa1p and used $NAD(P)^+$ as a cofactor. Finally, the intermediate compound is hydrolyzed back into 3HP and released glutathione by the S-formylglutathione hydrolase encoded by Yjl068C. The proposed glutathione-dependent cyclic mechanism of 3HP detoxification has provided new insights into molecular response to 3HP in *S. cerevisiae*.

Example 6. The role of GSH in 3HP detoxification pathway

The role of GSH in 3HP detoxification was investigated. The WT, SFA1-allles, and *sfa1Δ* strains were grown in the minimal medium with or without 50 g/L 3HP and supplemented with various concentration of external GSH (0-30 mM) and tested for 3HP tolerance. As seen in Figure 7, GSH addition (≥2.5 mM) restored the growth of the WT *S. cerevisiae* in the presence of 50 g/L 3HP up to the level slightly higher than that of the SFA1-alleles strains grown without GSH supplement. Furthermore, GSH addition has also improved the growth rate of the strain carrying the SFA1 alleles. The growth of the *sfa1Δ* strain could not be restored by addition of GSH, further supporting the proposed role of GSH in 3HP detoxification. In addition, GSH has no significant effect on all strains grown in the minimal medium without 50 g/L 3HP.

For testing 3HP tolerance phenotype, the pre-cultures were prepared by inoculation of a single colony in 1 mL Delft buffered (pH 3.5) media containing 10 g/L 3HP. The cultures were incubated at 30°C with 250 rpm overnight. Five µL of the overnight cultures were used to inoculate 100 µL Delft buffered (pH 3.5) with or without 50 g/L 3HP and various concentration of GSH (0, 1, 2.5, 5, 10, and 20 mM) in 96-well flat bottom plate. The 96-well plate was incubated at 30°C with shaking in the Synergy™ MX microplate reader (BioTek) and the absorbance was measured at 600 nm wavelength every 15 min for 42 hours. Experiments were done in triplicates.

Example 7. Enhanced 3HP tolerance in *S. cerevisiae* by improving glutathione production (prophetic).

From the previous example, addition of GSH enabled the viability the WT strain and improved the growth of the SFA1 allele's strains. However, the GSH addition is not economically suitable for industrial production of 3HP. Therefore, increasing intracellular pool of GSH by overexpression of genes involved in glutathione biosynthesis would be an alternative approach to reduce production cost.

In *S. cerevisiae*, GSH is synthesized by two consecutive ATP-dependent reactions. The γ-glutamyl-cysteine (γ-GC) synthetase (GCS,

EC 6.3.2.2) encoded by GSH1 catalyzes the conjugation of L-glutamic acid and L-cysteine into γ-GC. The glutathione synthetase (GS, EC 6.3.2.3) encoded by GSH2 (SEQ ID 12) catalyzes the synthesis of GSH from γ-GC and glycine (Grant *et al.*, 1997).

As the glutathione production requires three amnio acid substrates (L-cysteine, L-glutatmic acid and glycine), increasing the intracellular concentration of the substrates e.g. L-cysteine should improve the glutathione productivity. In this study, two genes in the cysteine biosynthetic pathway, *MET14* and *MET16*, were overexpressed in combination with *GSH1* and *GSH2* genes and the constructed strains were tested for the effect of excess intracellular GSH concentration on 3HP tolerance.

To construct the expression vector for glutathione production, the fragments carrying the *GSH1* (SEQ ID NO 11) and *GSH2* (SEQ ID NO 12) genes, and the *PTEF1-PPGK1* double promoter fragment (SEQ ID NO 13) containing the correct overhangs for USER cloning were generated by PCR amplification using primers and templates as indicated in Table 2. The three fragments were then ligated into pESC-URA-USER by USER cloning as previously described in example 3 to generate pESC-URA-GSH1-GSH2 plasmid.

To construct the expression vector for improving cysteine production, the fragments carrying the *MET14* (SEQ ID NO14) and *MET16* (SEQ ID NO15) genes containing the correct overhangs for USER cloning were generated by PCR amplification using primers and templates as indicated in Table 2. The final plasmid pESC-HIS-MET14-MET16 was constructed by ligation of *MET14*, *MET16* and PTEF1-PPGK1 fragments into pESC-HIS-USER by USER cloning as previously described in Example 3.

The pESC-URA-GSH1-GSH2 plasmid was co-transformed with either pESC-HIS or pESC-HIS-MET14-MET16 plasmids into different background strains (ST726, ST727, and ST728) using the lithium acetate transformation protocol. The cells were selected on SC-Ura-His-Leu media. The transformants were streak purified on SC-Ura-His-Leu plate in order to obtain single colonies.

The resulting strains were tested for 3HP tolerance phenotype as described in Example 2.

Example 8. Identification of point mutations in the Sfa1p protein that confers 3HP tolerance phenotype.

The two mutaions at Cys276 and Met283 residues in the Sfa1p (previously mentioned in Example 2) were investigated whether substitution of these amino acid residues with other amino acids apart from serine (Ser) and isoleucine (Ile), respectively, will also result in 3HP tolerance phenotype. Furthermore, the cysteine residues (Cys205, Cys237, and Cys290) in the Sfa1p were also selected for site-directed mutatgenesis and tested whether mutation in these residues will also improve 3HP tolerance in *S. cerevisiae*. Intitial results have not shown improvement by mutation at these sites. Thus,substitution of amino acids Cys205, Cys237 and Cys290 in the Sfa1p by Ser205, Ser237, and Ser290, respectively, did not result in 3HP tolerance phenotype in *S. cerevisiae*.

Substitution of the Cys276 residue with other amino acids e.g. threonine (Thr), glycine (Gly), valine (Val) and alanine (Ala) also gives 3HP tolerance phenotype in *S. cerevisiae*. In addition, the constructed strains where the Met283 residue in the Sfa1p was replaced with either Val or Ala also were aslo able to grow in the presence of 50 g/L 3HP. The growth of the resulting strains on minimal medium containing 50 g/L 3HP was shown in Figure 8.

The PCR site-directed mutagenesis was performed to replace the Cys276 residue with either Gly, Ala, Val or Thr using primers and templates as listed in Table 2. The Met283 was changed into either Val or Ala, whereas the Cys205, Cy237 and Cys290 residues were also substituted with Ser residue (Table 2). The PCR mix contained: 30 µl water, 10 µl high fidelity Phusion® polymerase buffer (5x), 2.5 µl 10 mM dNTP, 1 µl Phusion® polymerase, 1.5 µl forward primer at 10 µM concentration, 1.5 µl reverse primer at 10 µM concentration, 1.5 µl DNA template (200 ng), and 1.5 µl DMSO (100%). The cycling program was: 98°C for 2 min, 18 cycles of [98°C for 10 sec, 55°C for 30 sec, 68°C for 9 min], 68°C for 12 min, pause at 10°C. The PCR reaction was treated with 1 µl DpnI enzyme and incubated at 37°C for 1 hour to remove the plasmid template. The treated PCR samples were transformed into chemically competent *E. coli DH5alpha* and selected

on LB containing 100 µg/mL ampicillin. The plasmids were isolated from each *E. coli* transformants and the correct mutations in the *SFA1* gene were confirmed by sequencing using primer SFA1_U1_rv.

To generate the substrates for *SFA1* allele replacement, the upstream fragment for each SFA1 mutations was generated by fusion PCR using the SFA1-UP fragment, SFA1-allele fragment and the first 2/3 KlLEU2 fragment as templates and using primers KO_sfa1_fw and NB335KlLEURev1 (Table 2).

The strains carrying the point mutation in the *SFA1* gene were constructed by replacing the *KanMX* cassette in the *sfa1Δ* strain with the corresponding upstream fragment (Table 2) and downstream fragment (LEU2_U_2/3_END_SFA1_DOWN fragments) of the *SFA1* gene as previously mentioned in Example 3. The strains were selected on SC-Leu media. The transformants were streaked purified on SC-Leu to obtain single colonies. The correct transformants were confirmed by PCR analysis using primers KO_sfa1_test_fw and SFA1_U1-rv and the mutation in the SFA1 gene was verified by sequencing using primer SFA1_U1_rv.

The contructed strains were tested for 3HP tolerance phenotype as described in Example 3.

Example 9. Improving 3HP tolerance in other cells.

Based on the above examples, 3HP tolerance in *S. cerevisiae* is improved by increasing the availability of intracellular GSH either by addition of external GSH or improving GSH production. This example demonstrates that the GSH addition also helps to improve 3HP tolerance on other microorganisms e.g. *E. coli* and several yeast strains (*Schizosaccharomyces pombe*, *Saccharomyces kluyveri*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Yarrowia lipolytica*, *Cyberlindnera jadinii*, *Torulaspora delbrueckii*, *Rhodotorula minuta*).

As seen in Figure 9, addition of 5 mM GSH restored the viability of several yeast strains i.e. *S. cerevisiae*, *S. kluyveri* and *K. lactis* grown in the presence of 50 g/L 3HP. For some yeast strains e.g. *K. marxianus*, *Y. lipolytica*, *C. jadinii*, *S. pombe*, *T. delbrueckii* and *Rhodotorula minuta*, these strains could tolerate

more than 50 g/L 3HP with decreasing in the specific growth rate compared to the cells grown without the presence of 50 g/L 3HP.However, the specic growth rate of these strains grown in the presence of 50 g/L 3HP was improved by the addition of 5 mM GSH in the cultivation medium.

In Figure 10, addition of 5 mM GSH also restored the viability of four *E. coli* strains i.e. W3110, CROOKS, W and BL21 grown in M9 minimal medium supplemented with 20 g/L 3HP. For MG1655, the strain could grow in M9 medium containing 20 g/L 3HP with 2.2-fold decrease in the specific growth rate compared to the cells grown in M9 medium. However, the specic growth rate of MG1655 strain was improved by the addition of 5 mM GSH in the cultivation medium containing 20 g/L 3HP.

The results strongly supported that the proposed role of GSH in 3HP detoxification mechanism are similar in several microorganisms.

The yeast strains were grown in 3 mL Delft medium (pH 6.0) at 250 rpm, 25°C, overnight. Three µL of the overnight cultures were used to inoculate 100 µL Delft medium (pH 6.0) with or without 50 g/L 3HP in 96-well flat bottom plate. The 96-well plate was incubated with shaking in the Synergy™ MX microplate reader (BioTek) at 25°C and the absorbance was measured at 600 nm wavelength every 15 min for 72 hours. Experiments were done in triplicates, and the specific growth rate (h^{-1}) was calculated. The effect of GSH addition was performed by adding 5mM GSH into the cultivation medium containing 50 g/L 3HP. The growth of each strain was determined as mentioned above.

For *E. coli*, the effect of GSH addition on the growth of *E. coli* in the presence of 20 g/L 3HP was investigated. Five platform *E. coli* wild-type strains e.g. W3110, CROOKS, W, BL21 (DE3) and MG1655 were selected for 3HP tolerance experiment. Single colony from each strain was inoculated into 3 mL M9 medium and incubated at 250 rpm, 37°C, overnight. Two µL of the overnight cultures were used to inoculate 150 µL M9 medium, M9 with 20 g/L 3HP, and M9 with 20 g/L 3HP and 5 mM GSH in 96-well flat bottom plate. The pH of all *E. coli* tested media was at pH 7.0. The 96-well plate was incubated

with shaking in the ELx808™ Absorbance microplate reader (BioTek) at 37°C and the absorbance was measured at 630 nm wavelength every 5 min for 22 hours. Experiments were done in triplicates, and the specific growth rate (h^{-1}) was calculated.

The composition of M9 medium was as follows: 2.0 g/L glucose, 6.8 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl,0.24 g/L $MgSO_4$, 0.011 g/L $CaCl_2$, 0.5 mL/L trace elements solution, and 1.0 mL/L Wolfe's Vitamin solution. The trace elements solution included 1.0 g/L $FeCl_3.6H_2O$, 0.18 g/L $ZnSo_4.7H_2O$, 0.12 g/L $CuCl_2.2H_2O$, 0.12 g/L $MnSO_4.H_2O$, and 0.18 g/L $CoCl_2.6H_2O$. The Wolfe's vitamin solution included 10 mg/L pyridoxine hydrochloride, 5.0 mg/L thiamine_HCl, 5.0 mg/L riboflavin, 5.0 mg/L nicotinic acid, 5.0 mg/L calcium-(+) phantothenate, 5.0 mg/L para-amino benzoic acid, 5.0 mg/L thiotic acid, 2.0 mg/L d-biotin, 2.0 mg/L folic acid, and 0.1 mg/L vitamin B12. The trace elements and vitamin solutions were sterile-filtered and the solution was stored at 4°C. The M9 salt solution was prepared as a 10x concentrate solution and sterilized by autoclaving. 20% Glucose solution, 1M $MgSO_4$, and 1M $CaCl_2$ stock solutions were autoclaved separately.

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In this specification, unless expressly otherwise indicated, the word 'or' is used in the sense of an operator that returns a true value when either or both of the stated conditions is met, as opposed to the operator 'exclusive or' which requires that only one of the conditions is met. The word 'comprising' is used in the sense of 'including' rather than in to mean 'consisting of'. All prior teachings acknowledged above are hereby incorporated by reference. No acknowledgement of any prior published document herein should be taken to be an admission or representation that the teaching thereof was common general knowledge in Australia or elsewhere at the date hereof.

The invention may be summarised according to the following clauses:

1. A cell having a metabolic pathway producing 3-hydroxypropionic acid (3HP), said cell exhibiting tolerance for 3HP and having one or more genetic modifications that provide for an enhanced activity of 3HP detoxification by a reaction pathway that includes a glutathione-dependent dehydrogenase reaction.

2. A cell as defined in clause 1, wherein a said genetic modification is one or more mutations in a gene encoding a glutathione-dependent formaldehyde dehydrogenase conferring said tolerance.

3. A cell as defined in clause 2, wherein said one or more mutations are in a gene equivalent to *SFA1* of *Saccharomyces cerevisiae*.

4. A cell as defined in clause 3, wherein said one or more mutations are in a gene encoding a protein having the sequence SEQ ID NO 1 or a protein with more than 80% homology to SEQ ID NO 1.

5. A cell as defined in any one of clauses 2 to 4, wherein the mutation is at a position equivalent to the position aa276 Cys and/or at aa283 Met of the *SFA1* gene of *Saccharomyces cerevisiae*.

6. A cell as defined in clause 5, wherein said mutation in gene *SFA1* is Cys276->Ser, Cys276->Val, Cys276->Thr, Cys276->Gly, Cys276->Ala and/or Met283->Ile, Met283->Ala, Met283->Val.

7. A cell as defined in any preceding clause, wherein a said genetic modification produces overexpression of a native, or heterologous, or mutated glutathione-dependent formaldehyde dehydrogenase to confer said 3HP tolerance.

8. A cell as defined in clause 7, wherein said genetic modification produces overexpression of a native or heterologous glutathione-dependent formaldehyde dehydrogenase which has the sequence SEQ ID NO 1 or is a protein with more than 80% homology to SEQ ID NO 1.

9. A cell as defined in any preceding clause, wherein the cell is genetically modified for increased production of glutathione.

10. A cell as defined in clause 9, wherein the cell overexpresses the glutathione biosynthetic genes gamma-glutamylcysteine synthetase and glutathione synthetase.

11. A cell as defined in clause 9 or clause 10, wherein the cell overexpresses genes that enhance the production of amino acid precursors for glutathione biosynthesis.

12. A cell as defined in any preceding clause, wherein said metabolic pathway comprises the enzyme malonyl-CoA reductase and/or the enzyme malonyl-CoA reductase (malonate semialdehyde-forming) in combination with the enzyme 3-hydroxyisobutyrate dehydrogenase and/or the enzyme hydroxypropionate dehydrogenase.

13. A cell as defined in any one of clauses 1 to 11, wherein said metabolic pathway comprises a malonyl-CoA reductase gene and an acetyl-CoA carboxylase gene.

14. A cell as defined in clause 13, wherein said metabolic pathway comprises the malonyl-CoA reductase gene from *Chloroflexus aurantiacus* (CaMCR).

15. A cell as defined in clause 14, wherein said metabolic pathway comprises the malonyl-CoA reductase gene from *Chloroflexus aurantiacus (CaMCR)* and the acetyl-CoA carboxylase gene (*ACC1*) of *S. cerevisiae*.

16. A cell as defined in any one of clauses 1 to 11, wherein said metabolic pathway comprises beta-alanine pyruvate aminotransferase and/or gamma-aminobutyrate transaminase in combination with hydroxyisobutyrate dehydrogenase and/or hydroxypropionate dehydrogenase.

17. A cell as defined in clause 16, wherein said metabolic pathway comprises the beta-alanine pyruvate aminotransferase from *Bacillus cereus* together with one

of the following: 3-hydroxypropanoate dehydrogenase from *Metallosphaera sedula*, 3-hydroxypropanoate dehydrogenase from *Sulfolobus tokadaii*, 3-hydroxypropanoate dehydrogenase from *E. coli* (YdfGp), 3-hydroxypropanoate dehydrogenase from *E. coli* (RutEp), 3-hydroxyisobutyrate dehydrogenase from *Pseudomonas aeruginosa*, 3-hydroxyisobutyrate dehydrogenase from *P. putida*, 3-hydroxyisobutyrate dehydrogenase from *Bacillus cereus*, or 3-hydroxyisobutyrate dehydrogenase from *Candida albicans*.

18. A cell as defined in any one of clauses 1 to 11, wherein said metabolic pathway comprises glycerol dehydratase and alcohol dehydrogenase.

19. A cell as defined in any one of clauses 1 to 11, wherein said metabolic pathway comprises lactate dehydrogenase, propionate CoA-transferase, lactoyl-CoA dehydratase, enoyl-CoA hydratase and 3-hydroxyisobutyryl-CoA hydrolase.

20. A cell as defined in any preceding clause, wherein said cell is a yeast cell.

21. A cell as defined in clause 18, wherein the yeast cell is of the genus *Saccharomyces*, *Schizosaccharomyces*, *Kluyveromyces*, *Pichia*, *Candida*, *Yarrowia*, *Brettanomyces*, *Hansenula*, *Lipomyces*, and *Issatchenkia*.

22. A cell as defined in clause 18, wherein the yeast cell is a *Saccharomyces cerevisiae* cell.

23. A cell as defined in clause 22, wherein said genetic modification comprises one or more mutations in gene *SFA1*

at aa276 Cys and/or at aa283 Met.

24. A cell as defined in any one of clauses 1 to 19, wherein said cell is a bacterial cell.

25. A cell as defined in any one of clauses 1 to 19, wherein said bacterial cell is of the genus *Eschericia*, *Lactobacillus*, *Lactococcus*, *Corynebacterium*, *Clostridium*, or *Bacillus*.

26. A cell as defined in any one of clauses 20 to 25, wherein the cell is *S. cerevisiae*, *S. pombe*, *S. kluyveri*, *K. lactis*, *K. marxianus*, *Y. lipolytica*, *T. delbreueckii*, *R. minuta*, *I. orientalis*, *P. stipites*, *L. starkeyi*, *C. guilliermondii*, or *E. coli*.

27. A yeast exhibiting tolerance for 3HP, said yeast having a mutation in gene *SFA1* conferring said tolerance.

28. A yeast as defined in clause 27, wherein the yeast is *Saccharomyces cerevisiae* and and said mutation in gene *SFA1* is at aa276 Cys and/or at aa283 Met.

29. A yeast as defined in clause 28, wherein said mutation in gene *SFA1* is Cys276->Ser, Cys276->Val, Cys276->Thr, Cys276->Gly, Cys276->Ala and/or Met276->Ile, Met276->Ala, Met276->Val

30. A yeast as defined in clause 29, wherein said mutation in gene *SFA1* is Cys->Ser(aa276) and/or Met->Ile(aa283).

31. A method of producing 3HP comprising cultivating a 3HP producing cell under 3HP producing conditions in a culture medium so as to produce 3HP, wherein toxicity of 3HP is reduced by an enhanced activity of 3HP detoxification by a reaction pathway that includes a glutathione-dependent dehydrogenase reaction.

32. A method as defined in clause 31, wherein said culture medium is supplemented with glutathione.

33. A method as defined in clause 32, wherein glutathione is added to the culture medium to produce a concentration therein of >2.5 mM.

34. A method as defined in clause 31 or clause 32, wherein said cell has a genetic modification conferring an enhanced glutathione production ability thereon.

35. A method as defined in clause 34, wherein the cell overexpresses the glutathione biosynthetic genes gamma-glutamylcysteine synthetase and glutathione synthetase.

36. A method as defined in clause 34 or clause 35, wherein the cell overexpresses genes that enhance the production of amino acid precursors for glutathione biosynthesis.

37. A method as defined in any one of clauses 31 to 36, wherein a concentration of 3HP in excess of **1 g/l** is produced in said culture medium.

38. The use of an enhanced glutathione-dependent dehydrogenase reaction in a cell to enhance tolerance of

said cell to 3HP.

39. A use as defined in clause 38, wherein said reaction is one converting S-(3-hydroxypropanoyl)glutathione to S-(3-ketopropanoyl)glutathione.

Claims:

1. A cell having a metabolic pathway producing 3-hydroxypropionic acid (3HP), said cell exhibiting tolerance for 3HP and having one or more genetic modifications that provide for an enhanced activity of 3HP detoxification by a reaction pathway that includes a glutathione-dependent dehydrogenase reaction.

2. A cell as claimed in claim 1, wherein a said genetic modification is one or more mutations in a gene encoding a glutathione-dependent formaldehyde dehydrogenase conferring said tolerance.

3. A cell as claimed in claim 1 or claim 2, wherein said one or more mutations are in a gene encoding a protein having the sequence SEQ ID NO 1 or a protein with more than 80% homology to SEQ ID NO 1.

4. A cell as claimed in claim 2 or claim 3, wherein the mutation is at a position equivalent to the position aa276 Cys and/or at aa283 Met of the *SFA1* gene of *Saccharomyces cerevisiae*.

5. A cell as claimed in claim 4, wherein said mutation in gene *SFA1* is Cys276->Ser, Cys276->Val, Cys276->Thr, Cys276->Gly, Cys276->Ala and/or Met283->Ile, Met283->Ala, Met283->Val.

6. A cell as claimed in any preceding claim, wherein a said genetic modification produces overexpression of a native, or heterologous, or mutated glutathione-dependent

formaldehyde dehydrogenase to confer said 3HP tolerance.

7. A cell as claimed in any preceding claim, wherein the cell is genetically modified for increased production of glutathione.

8. A cell as claimed in claim 7, wherein the cell overexpresses genes that enhance the production of amino acid precursors for glutathione biosynthesis.

9. A cell as claimed in any preceding claim, wherein said metabolic pathway comprises the enzyme malonyl-CoA reductase and/or the enzyme malonyl-CoA reductase (malonate semialdehyde-forming) in combination with the enzyme 3-hydroxyisobutyrate dehydrogenase and/or the enzyme hydroxypropionate dehydrogenase, or wherein said metabolic pathway comprises a malonyl-CoA reductase gene and an acetyl-CoA carboxylase gene, or wherein said metabolic pathway comprises beta-alanine pyruvate aminotransferase and/or gamma-aminobutyrate transaminase in combination with hydroxyisobutyrate dehydrogenase and/or hydroxypropionate dehydrogenase or wherein said metabolic pathway comprises glycerol dehydratase and alcohol dehydrogenase or wherein said metabolic pathway comprises lactate dehydrogenase, propionate CoA-transferase, lactoyl-CoA dehydratase, enoyl-CoA hydratase and 3-hydroxyisobutyryl-CoA hydrolase.

10. A cell as claimed in any preceding claim, wherein said cell is a yeast cell or is a bacterial cell.

11. A method of producing 3HP comprising cultivating a 3HP producing cell under 3HP producing conditions in a

culture medium so as to produce 3HP, wherein toxicity of 3HP is reduced by an enhanced activity of 3HP detoxification by a reaction pathway that includes a glutathione-dependent dehydrogenase reaction.

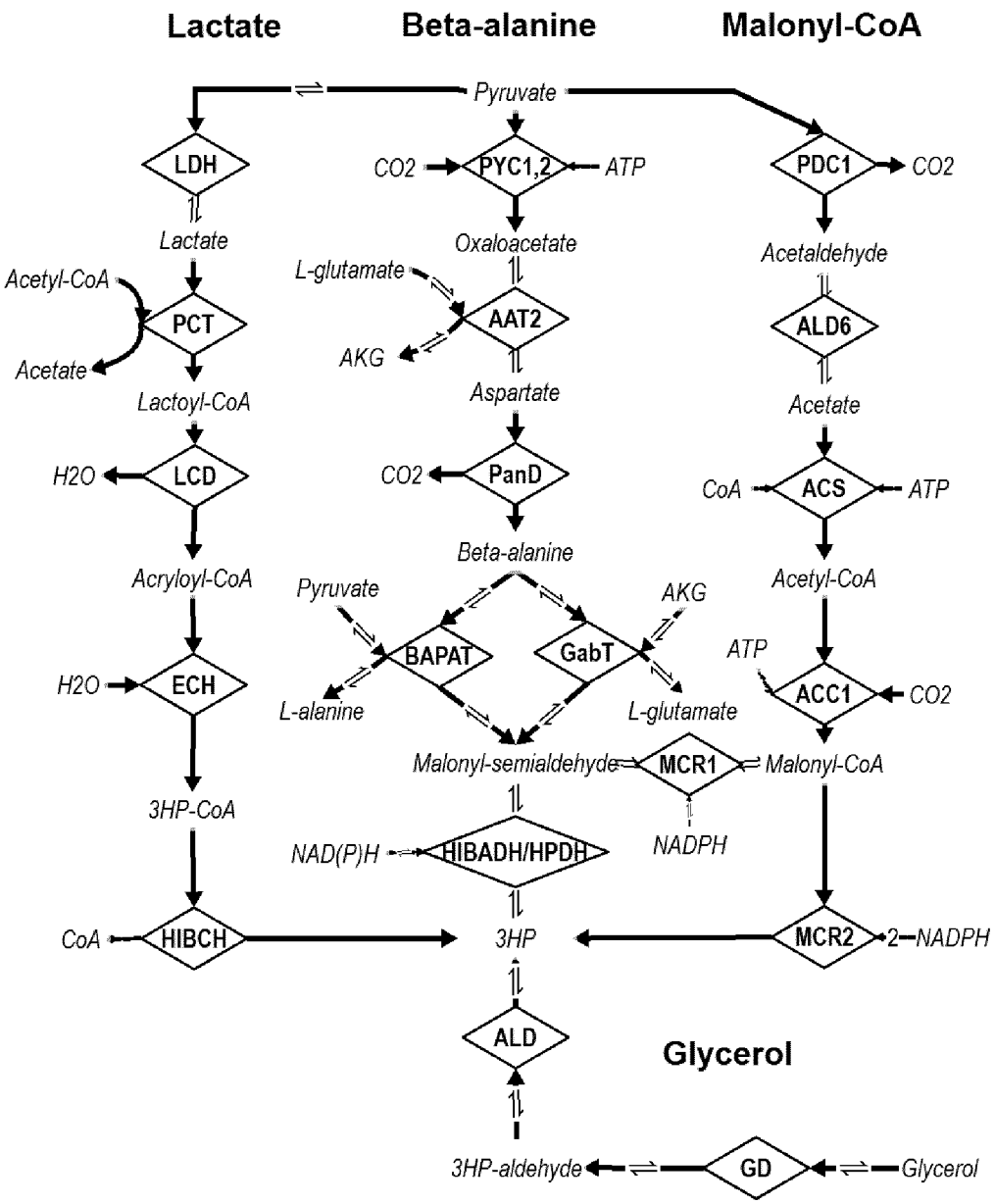
12. A method as claimed in claim 11, wherein said culture medium is supplemented with glutathione.

13. A method as claimed in claim 11 or claim 12, wherein the cell overexpresses the glutathione biosynthetic genes gamma-glutamylcysteine synthetase and glutathione synthetase.

14. A method as claimed in any one of claims 11 to 13, wherein the cell overexpresses genes that enhance the production of amino acid precursors for glutathione biosynthesis.

15. The use of an enhanced glutathione-dependent dehydrogenase reaction in a cell to enhance tolerance of said cell to 3HP.

Figure 1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Beta-alanine
Malonyl-CoA
Pyruvate
LDH
CO2
PYC1,2
ATP
PDC1
CO2
Lactate
Oxaloacetate
Acetaldehyde
Acetyl-CoA
L-glutamate
AAT2
ALD6
PCT
AKG
Acetate
Aspartate
Acetate
Lactoyl-CoA
H2O
LCD
CO2
PanD
CoA
ACS
ATP
Beta-alanine
Acryloyl-CoA
Pyruvate
AKG
Acetyl-CoA
BAPAT
GabT
ATP
H2O
ECH
ACC1
CO2
L-alanine
L-glutamate
Malonyl-semialdehyde
MCR1
Malonyl-CoA
3HP-CoA
NAD(P)H
HIBADH/HPDH
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ALD
Glycerol
3HP-aldehyde
GD
Glycerol

Figure 2.

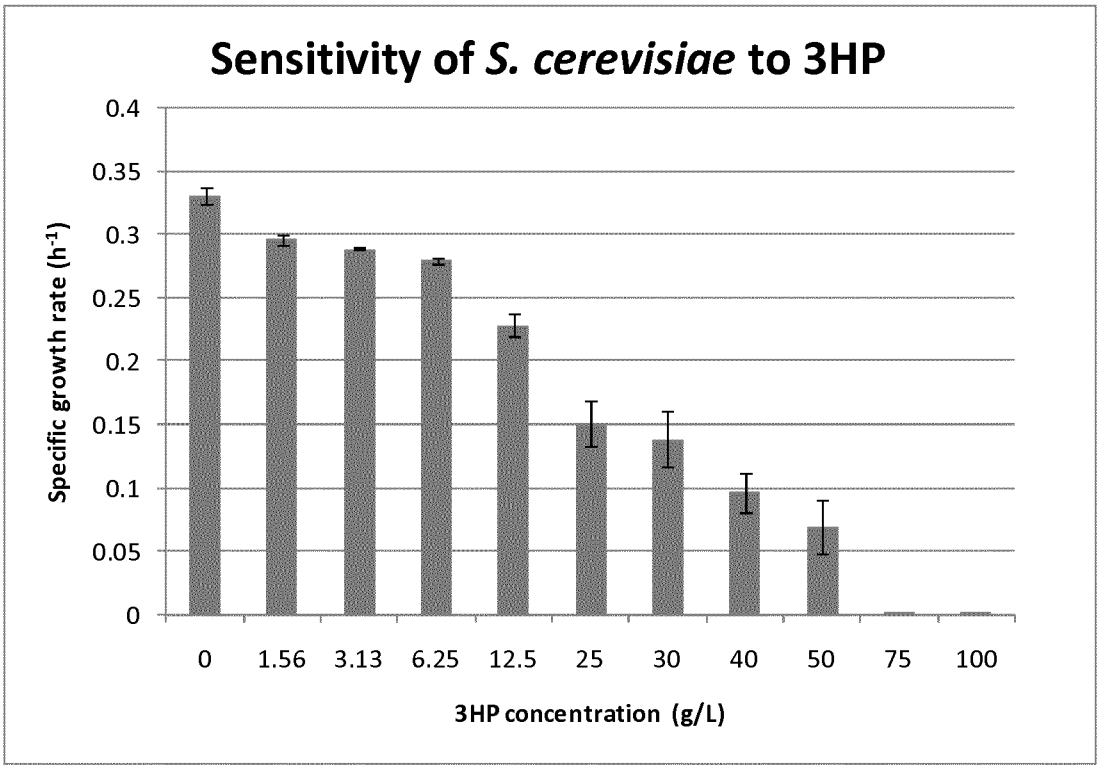
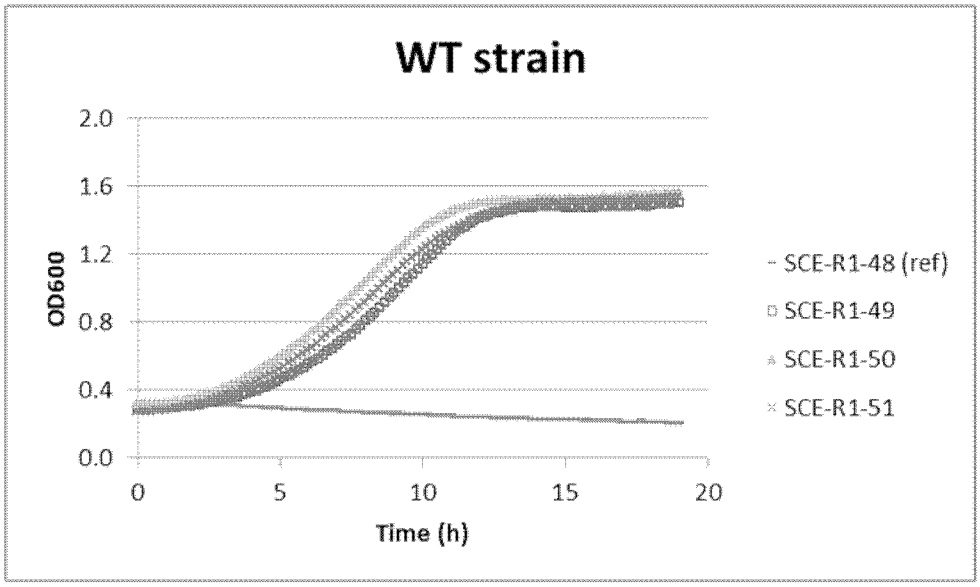

Sensitivity of S. cerevisiae to 3HP
Specific growth rate (h-1)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1.56
3.13
6.25
12.5
25
30
40
50
75
100
3HP concentration (g/L)

Figure 3.


WT strain
OD600
2.0
1.6
1.2
0.8
0.4
0.0
0
5
10
15
20
Time (h)
SCE-R1-48 (ref)
SCE-R1-49
SCE-R1-50
SCE-R1-51

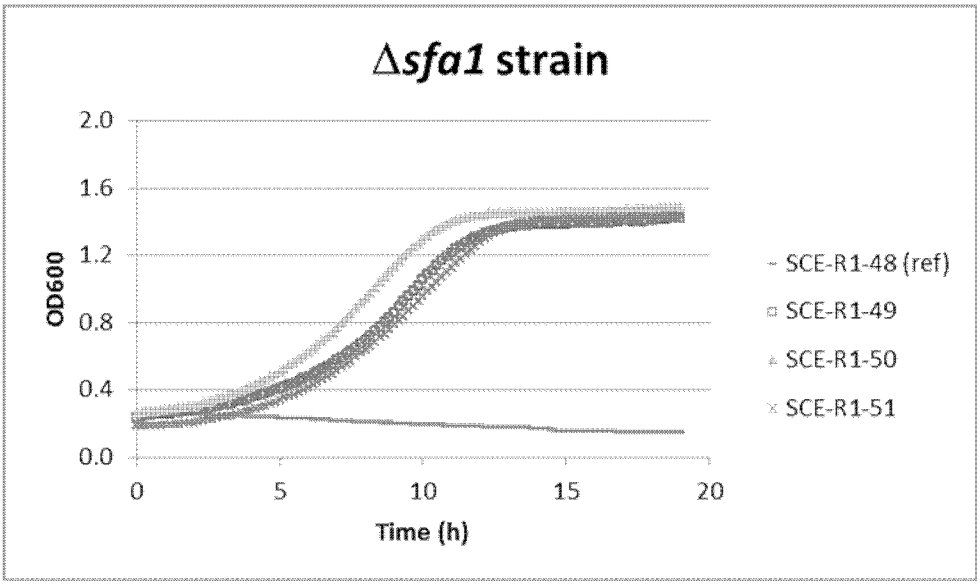

Δsfa1 strain
OD600
2.0
1.6
1.2
0.8
0.4
0.0
0
5
10
15
20
Time (h)
SCE-R1-48 (ref)
SCE-R1-49
SCE-R1-50
SCE-R1-51

Figure 4.

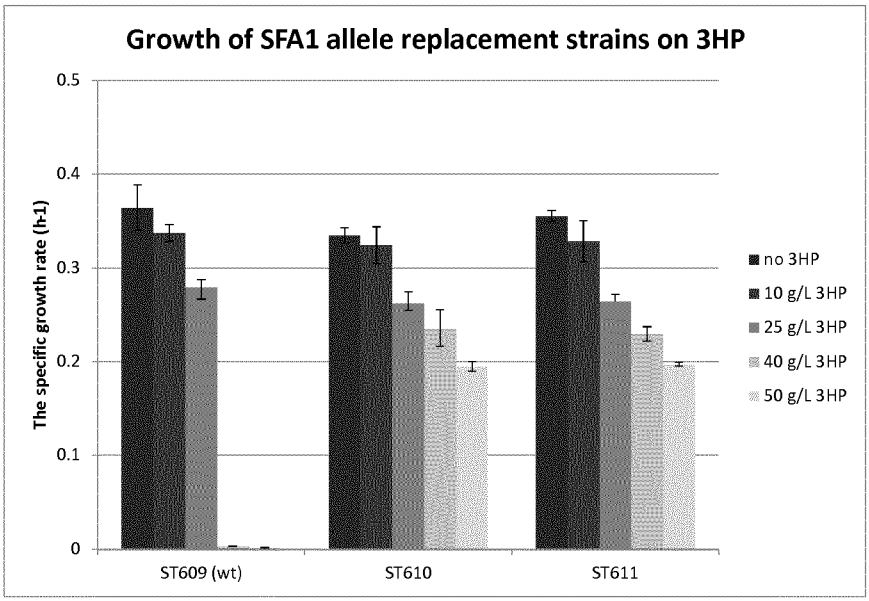
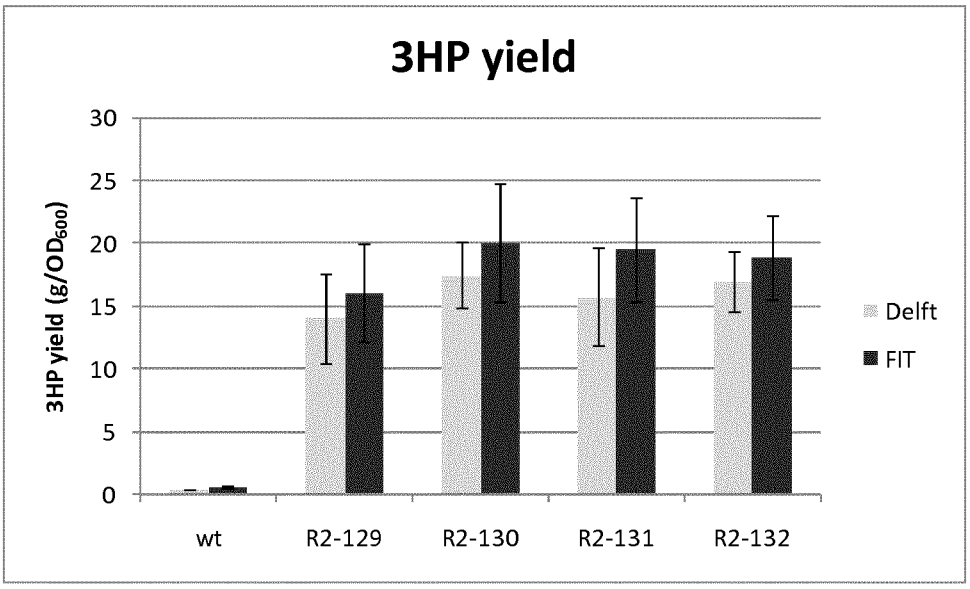


Figure 5.



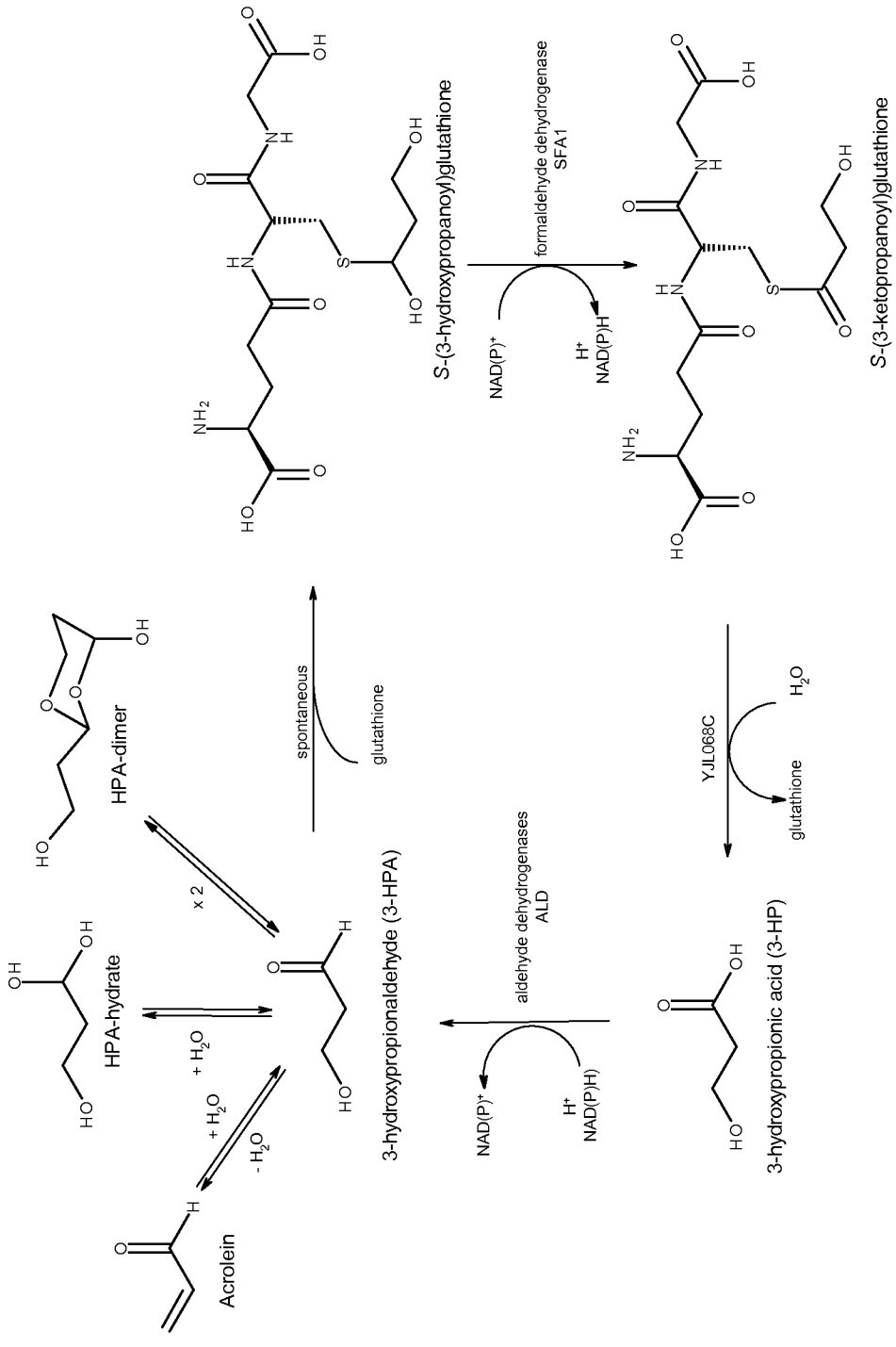

Acrolein
HPA-hydrate
HPA-dimer
3-hydroxypropionaldehyde (3-HPA)
3-hydroxypropionic acid (3-HP)
S-(3-hydroxypropanoyl)glutathione
S-(3-ketopropanoyl)glutathione
+ H2O
- H2O
x 2
spontaneous
glutathione
aldehyde dehydrogenases
ALD
NAD(P)+
H+
NAD(P)H)
YJL068C
H2O
glutathione
formaldehyde dehydrogenase
SFA1
NAD(P)+
H+
NAD(P)H

Figure 6.

Figure 7.

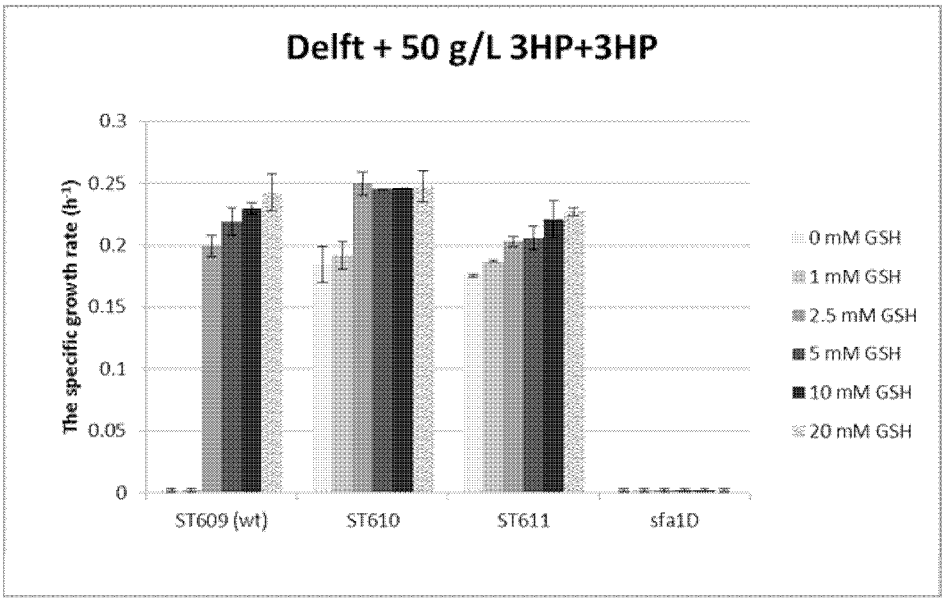


Figure 8.

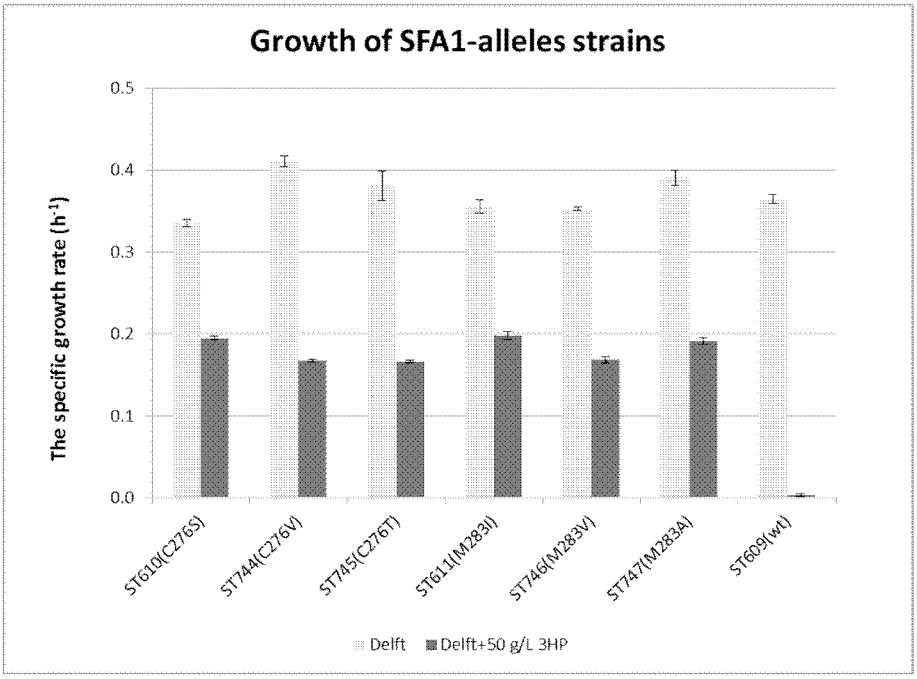


Figure 9.

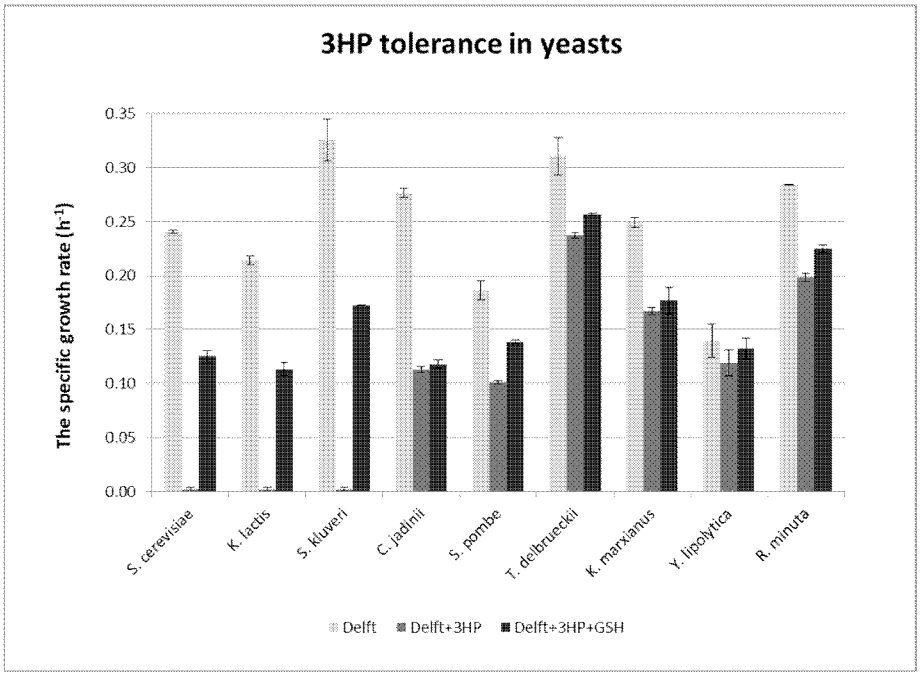
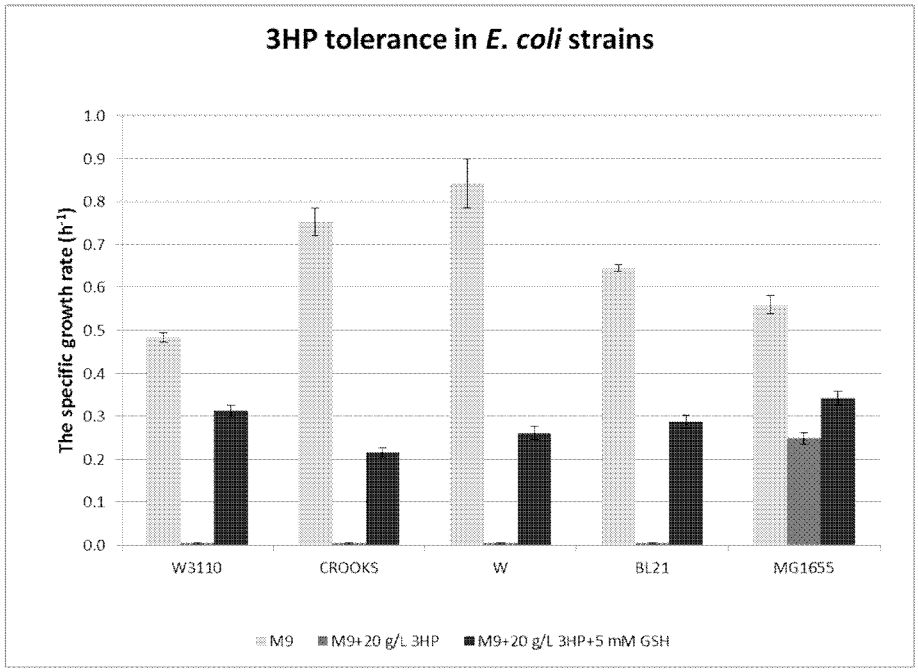


Figure 10.



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/062267

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/02 C12N9/00 C12P7/42 C12P7/52 C12N9/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2010/011874 A2 (OPX BIOTECHNOLOGIES INC [US]; UNIV COLORADO [US]; WARNECKE-LIPSCOMB TA) 28 January 2010 (2010-01-28) the whole document	1-15
A	YASOKAWA D ET AL: "Toxicogenomics using yeast DNA microarrays", JOURNAL OF BIOSCIENCE AND BIOENGINEERING, ELSEVIER, AMSTERDAM, NL, vol. 110, no. 5, 1 November 2010 (2010-11-01), pages 511-522, XP027447194, ISSN: 1389-1723, DOI: 10.1016/J.JBIOSC.2010.06.003 [retrieved on 2010-07-10] the whole document	1-15

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 July 2014	07/08/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Roscoe, Richard

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/062267

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	VINOD KUMAR ET AL: "Recent advances in biological production of 3-hydroxypropionic acid", BIOTECHNOLOGY ADVANCES, vol. 31, no. 6, 1 November 2013 (2013-11-01), pages 945-961, XP055093428, ISSN: 0734-9750, DOI: 10.1016/j.biotechadv.2013.02.008 the whole document -----	1-15

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/062267

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010011874 A2	28-01-2010	AU 2009273953 A1	28-01-2010
		CA 2731509 A1	28-01-2010
		CN 102317436 A	11-01-2012
		EP 2318514 A2	11-05-2011
		US 2011244575 A1	06-10-2011
		WO 2010011874 A2	28-01-2010